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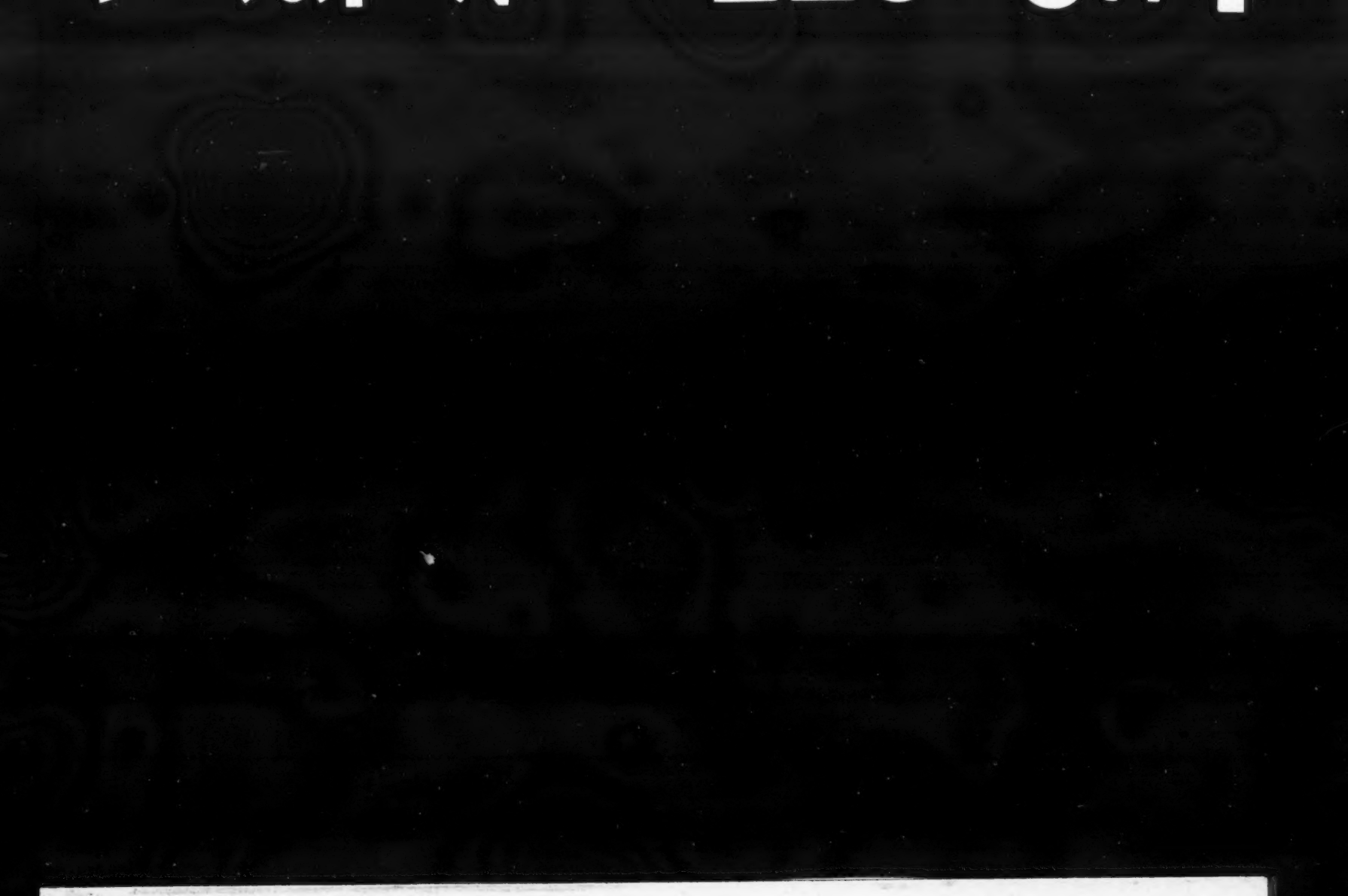

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THE ACTION OF FUMIGANTS ON INSECTS

III. THE FATE OF HYDROGEN CYANIDE IN SITOPHILUS GRANARIUS (L.)[1]

E. J. BOND

Abstract

A study of the fate of hydrogen cyanide in fumigated *S. granarius* adults, using carbon-14 labelled cyanide, showed that this poison became generally distributed and combined with various metabolites. Labelled carbon was found in three compounds of a trichloroacetic acid extract and in one compound of the hydrolyzed proteins and in the body fats. Only a very small amount of the carbon was excreted from the insect's body as carbon dioxide, but a considerable amount was found in the excrement; nine radioactive compounds were isolated from the water-soluble fraction of the excrement. One of these, a polypeptide, contained nearly half of the total labelled carbon that was excreted and most of the activity was present in the aspartic acid portion of the compound; thus it appears that this insect can not only excrete amino acids but also it can synthesize and use them for the elimination of cyanide from their bodies.

Introduction

The fate of hydrogen cyanide in insects is less well understood than is its fate in mammals. In the latter it combines with thiosulphates in the presence of rhodanese to form the less toxic thiocyanate (1) and with cystine to form 2-iminothiazolidine-4-carboxylic acid (2). The carbon from radioactive cyanide injected into rats has been found in several compounds such as cyanocobalamin, allantoin, choline, methionine, and carbon dioxide (3). Although insects can tolerate and recover from much larger doses of cyanide than mammals, the detoxifying processes which remove cyanide from mammals could not be found in insects (4). In this investigation the distribution and fate of carbon-14 hydrogen cyanide were studied to determine how insects detoxify and eliminate cyanide from their bodies.

[1]Manuscript received May 8, 1961.
Contribution No. 198 from the Research Institute, Canada Department of Agriculture, University Post Office, London, Ontario. Some of this work was carried out at Imperial College of Science and Technology where it formed part of a thesis approved by the University of London for the Ph.D. degree.

Experimental

Adult *Sitophilus granarius* (L.) in 1-g quantities were exposed to carbon-14 hydrogen cyanide (8 mg/l. for 4 hours) in the apparatus previously described (5). After treatment the insects were divided into groups of 0.5 g each; one group was examined immediately and the survivors of the other group were examined 96 hours later. The distribution of cyanide in the insects' bodies was studied by determining the carbon-14 content of the cuticular waxes, the trichloroacetic acid (TCA) soluble compounds, the ether-soluble compounds, the proteins, and residual materials.

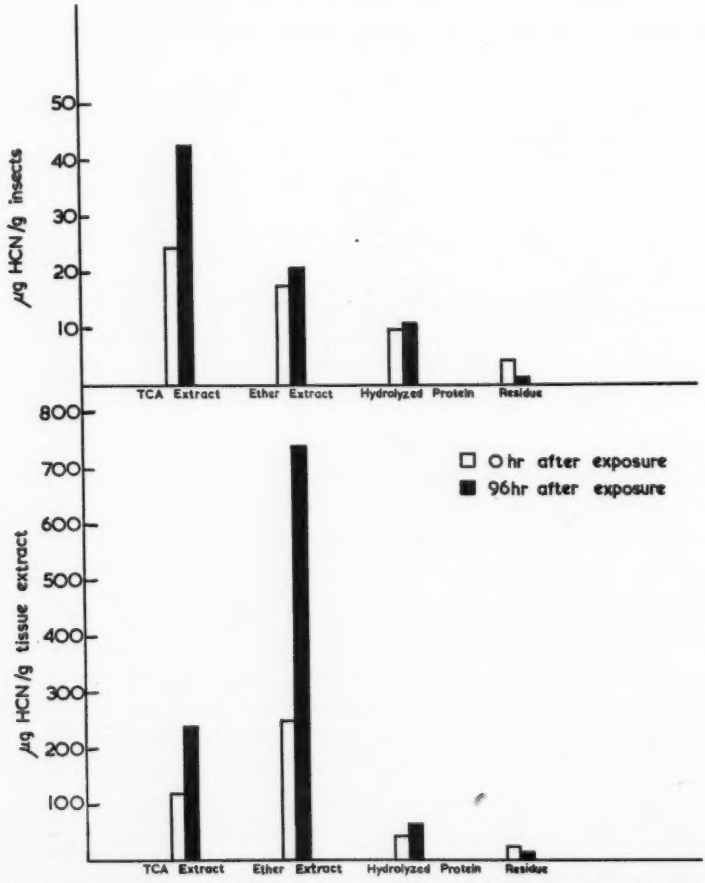


FIG. 1. The distribution of $HC^{14}N$ in extracts from *S. granarius*, immediately after, and 96 hours after, treatment.

The cuticular waxes were removed by shaking whole insects repeatedly in small volumes of ethyl ether. Aliquots of the dissolved waxes were then evaporated on filter paper disks for measurement of their radioactive content. The TCA-soluble compounds were extracted from homogenized insects and applied to filter paper disks on a planchette for counting in the Geiger counter. Similarly the ether-soluble compounds and the hydrolyzed proteins were removed and along with the remaining insoluble materials were made up into counting samples. The end products of metabolism, namely the faeces and the expired carbon dioxide from surviving insects, were also examined for labelled carbon. Carbon dioxide was collected in sodium hydroxide, and converted to barium carbonate for the preparation of counting samples. The radioactivity of all samples was measured with a Geiger counter having a thin end-window tube.

Distribution of Cyanide in the Insects

The total amount of cyanide in the insects, accounted for by radiometric analysis, was 56.7 μg/g in those insects analyzed immediately after exposure and 75.5 μg/g, 96 hours after exposure. In previous work (4) it was found that the average amount of cyanide absorbed by *S. granarius* during a 4-hour treatment was 172 μg/g and about 50% of this was heat labile and could be removed by hot aeration. The cyanide accounted for here, 33% and 44% respectively of the average uptake, was heat stable and represented cyanide that had irreversibly combined with components of the insects, possibly through a detoxication process. It is noteworthy that this fraction of the absorbed fumigant increased in quantity by 11% during the 4-day interval after treatment, the time during which the insects resumed respiration and recovered from paralysis.

The distribution of cyanide carbon in the various extracts is shown in Fig. 1 (the cyanide absorbed by the cuticular waxes is not represented in this figure because less than 1% was present in the waxes). The high content of carbon-14 in the TCA and ether extracts at 96 hours after exposure may reflect the importance of these fractions in a detoxication process.

Elimination of Carbon-14 from the Insects

Examination of the end products of carbon excretion, the expired carbon dioxide and the faeces, revealed that labelled carbon was excreted by two pathways. In the expired carbon dioxide the total amount of radioactive carbon eliminated in the first 4 days after fumigation represented about 0.4% of the total hydrogen cyanide absorbed and most of this was given off in the first 30 hours (Fig. 2). However, the presence of activity in the carbon dioxide indicated that some hydrogen cyanide entered into general metabolic reactions. In studies on the metabolism of cyanide in rats Boxer and Rickards (3) found that less than 0.1% of the carbon-14 from injected sodium cyanide was expired as carbon dioxide and the shape of the curve representing rate of elimination was similar to that shown here.

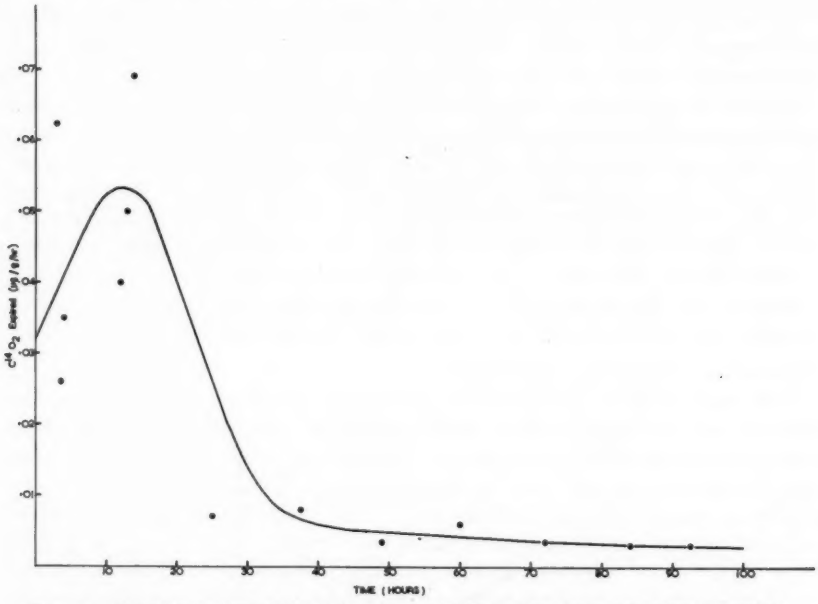


FIG. 2. The elimination of labelled carbon as $C^{14}O_2$ during the postfumigation period.

Radiometric analysis of the faeces collected from two batches of treated insects gave the following results:

Batch No.	Period of collection (hours)	Equivalent content of HCN (μg/g insects)	
		Water-soluble	Water-insoluble
1	92	31.2	1.3
2	102	37.7	0.4

The radioactive carbon in the water-soluble fraction of the faeces represented about 20% of the total amount of hydrogen cyanide originally absorbed. The presence of only small amounts of carbon-14 in the insoluble fraction indicated that uric acid, one of the main excretory products of insects, was not important in the elimination of the carbon from cyanide.

The Isolation of Metabolic Products Containing Carbon-14 by Paper Chromatography

The various extracts from fumigated insects were chromatographed to isolate compounds containing radioactive carbon and to determine whether any of these compounds corresponded to cyanide detoxication products found in mammals. The components of the TCA extract, the ether extract, and the

hydrolyzed proteins were separated by two-dimensional chromatography on 18-in. sq. sheets of Whatman No. 1 paper. The first solvent system, *n*-butanol, ethyl methyl ketone, ammonium hydroxide, and water (5:3:1:1), was run for 30 hours in the machine direction of the paper and the second solvent system, the organic phase of *n*-butanol, glacial acetic acid, and water (4:1:5), was run in a direction perpendicular to the first. Autoradiographs were prepared by placing the dried chromatograms in intimate contact with 14×17 in. Ilford "Industrial G" X-ray films for at least 12 days. After location of the active spots, various tests were applied to identify, or at least partially classify, the compounds. As well as the ninhydrin test, the following reagents were also used: mercuric acetate and diphenyl carbazone to test for uric acid and other purines (6), diazotized sulphanilic acid to test for 2-iminothiazolidine-4-carboxylic acid (2), and ferric nitrate to test for thiocyanates. The chromatograms were also examined under ultraviolet light for the presence of fluorescent compounds.

Figure 3 shows three radioactive ninhydrin-negative spots in the TCA extract (spots No. 3, 11, 13). These spots also gave negative tests for the thiocyanate radicle, purines, and 2-iminothiazolidine-4-carboxylic acid and they did not fluoresce in ultraviolet light. L-Glutamic acid was used as a marker in the second solvent and spot No. 14, which was inactive, was identified by its position and color as proline.

When the ether extract was chromatographed in these solvents none of the active compounds moved from the origin. The hydrolyzed proteins contained one weakly active compound that is shown as spot No. 1 (Fig. 4). Its position on the chromatogram corresponded with that of aspartic acid.

The water-soluble extract of the faeces from treated insects was chromatographed using butanol – acetic acid as the first solvent and buffered phenol–cresol as the second, according to the method of Levy and Chung (7). The distribution of the compounds in the extract is shown in Fig. 5 and the following were identified by their position on the chromatogram:

Spot No.	Compound	Spot No.	Compound
1	Cysteic acid and cystine	13	Glycine
8	Aspartic acid	18	Alanine
10	Glutamic acid	20	Histidine
12	Serine	21	Arginine
		25	Proline

An autoradiograph of the chromatogram indicated nine radioactive spots, only two of which were ninhydrin-positive (No. 9 and No. 13), and these are shown on the figure as stippled spots. The relative amount of radioactivity in each spot as compared with the total initially applied to the chromatogram was as follows:

Spot No.	2	5	7	9	13	14	16	19	24
% activity	3.6	4.0	6.1	46.1	1.5	2.7	1.8	3.6	3.0

Spot No. 13 which contained 1.5% of the total activity was identified by its position as glycine.

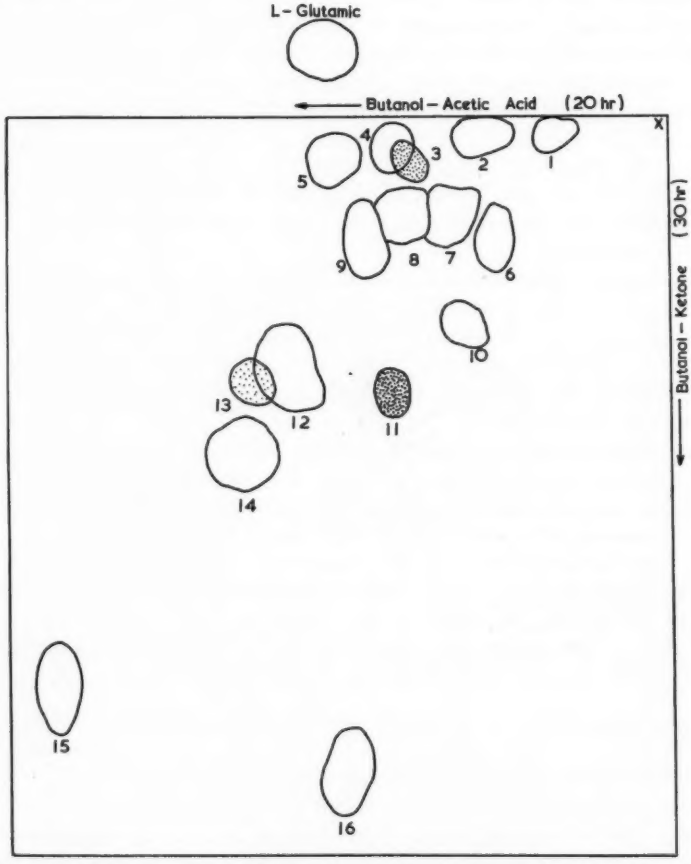


FIG. 3. Chromatogram of 10% trichloroacetic acid extract of fumigated insects.

The compound present in spot No. 9 was concentrated and purified by streaking water-soluble faeces extract across one side of a sheet of chromatography paper and developing the chromatogram in the butanol – acetic acid – water solvent. Then the streak representing spot No. 9 was located by autoradiography and removed from the chromatogram by cutting out the strip of paper and eluting with water. The eluate was further purified by rechroma-

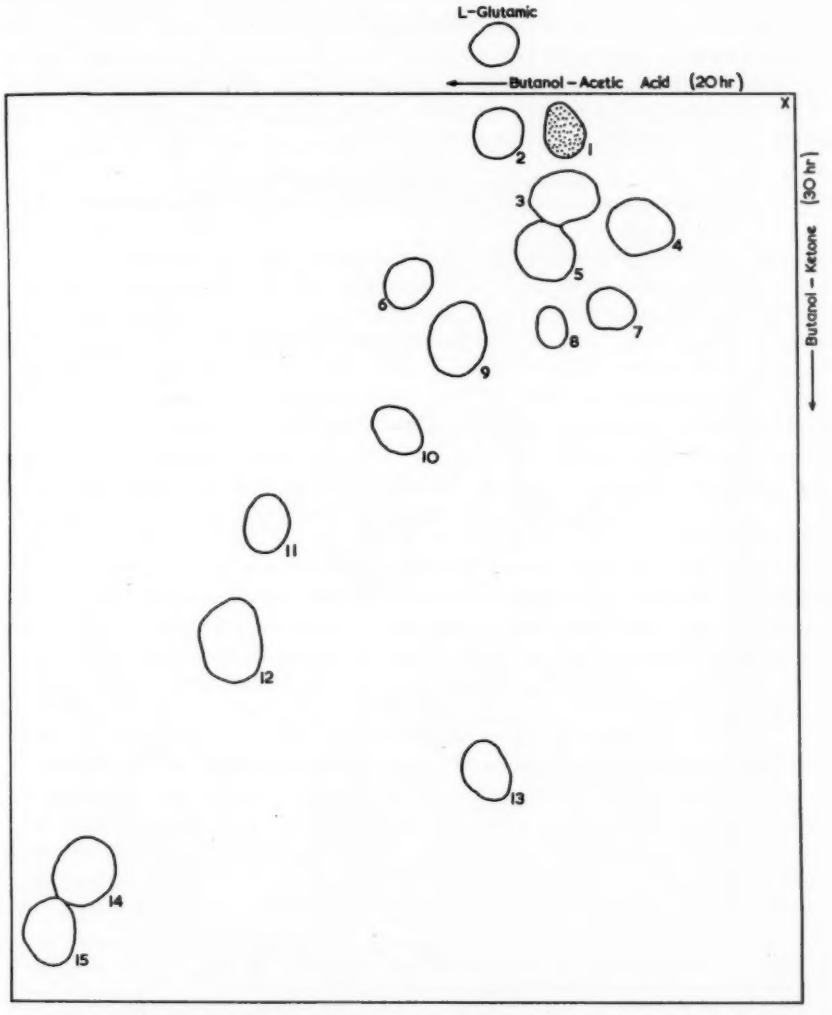


FIG. 4. Chromatogram of hydrolyzed proteins from fumigated insects. Hydrolysis of proteins was carried out by heating with 6 *N* HCl at 110° C in a sealed tube for 24 hours.

tographing in the phenol–cresol solvent and then tested for purity by two-dimensional chromatography in these solvents and in another set of solvents consisting of tertiary butyl alcohol, butanone, ammonia, and water (10:10:3:5) and methyl alcohol, pyridine, and water (20:1:5). The eluate was then subjected to acid hydrolysis (6 *N* HCl in a sealed tube at 110° C for 6 hours) and then chromatographed to determine the composition of spot No. 9. Using

the two different sets of solvents four ninhydrin-positive spots and one ninhydrin-negative spot were located and two of these spots were identified by their positions and colors as aspartic and glutamic acids. Autoradiography of these chromatograms showed that all of the activity was contained in the aspartic acid and the ninhydrin-negative spot.

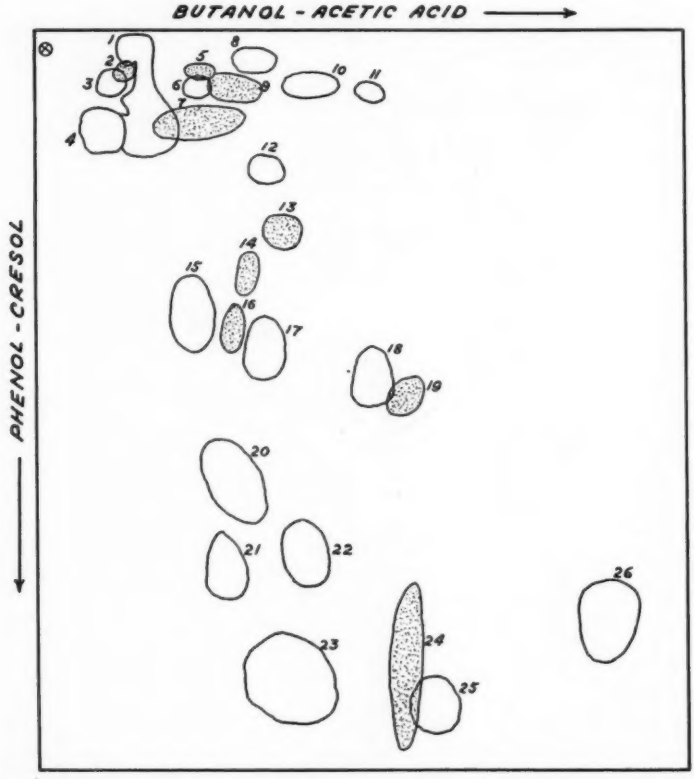


FIG. 5. Chromatogram of the water-soluble components of the excrement (stippled spots indicate radioactive compounds).

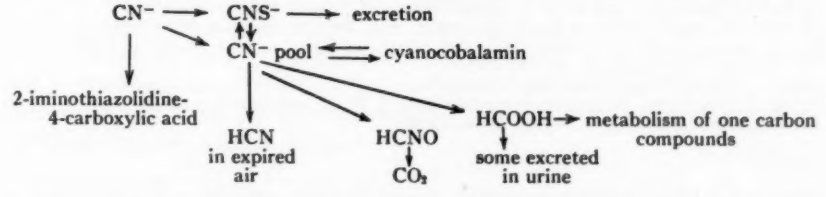
The radioactive aspartic acid was further studied to determine the location of the carbon-14 in the molecule. Using the method of Hallowes and Winteringham (8) the aspartic acid was decarboxylated and the radioactivity of the liberated carbon dioxide was measured. Eighty per cent of the activity was recovered as carbon dioxide. This figure agrees closely with the results of Hallowes and Winteringham when they used glutamic acid labelled on the 1-carboxyl carbon and thus indicates that all of the cyanide carbon was present in the 1-carboxyl group of the aspartic acid from *S. granarius*.

Discussion

The general distribution of cyanide throughout the insects points up its lack of specificity in biological reactions and, indeed, provides conditions whereby it may act in several ways at once. Winteringham and Lewis (9) have pointed out that insecticidal action may be caused by the particular combination of biochemical and physiological consequences of multiple action rather than by some unique single lesion and this may well be true of the action of cyanide on insects.

Cyanide inhibits a large variety of enzymes such as hemoproteins, copper, and zinc containing enzymes and enzymes depending on phosphopyridoxal or on disulphide groups and it may inhibit these enzymes by several different mechanisms. It may combine with an essential metal in the enzyme; it may remove a metal from the enzyme as an inactive complex; it may combine with a carbonyl group in the enzyme itself, or in a cofactor, or prosthetic group, or even in the substrate. Cyanide can also act as a reducing agent to break essential disulphide links in enzymes or it may act in still other ways as in the slow progressive inhibitions (10). Cyanide may also activate proteolytic enzymes to aid in the breakdown of tissues (11). The effect of cyanide on the respiration of *S. granarius*, its ability to combine both reversibly and irreversibly with many body components, its presence in the expired carbon dioxide and in many compounds of the faeces indicate the possibility of multiple action in the toxicity of cyanide to this insect. This possibility stands in sharp contrast to its action on mammals where death may be caused by interference with a single vital system. Previous results obtained using *S. granarius* (12) have suggested that respiratory inhibition, as produced by cyanide, may not be directly related to the lethal action of cyanide on this insect.

Neither of the cyanide detoxifying mechanisms of mammals were found in *S. granarius* but rather cyanide was incorporated into many compounds. In mammals cyanide has been found in several compounds other than those involved in single-carbon metabolism. This, however, accounts for only a very minor fraction of the cyanide injected into the animal. A scheme indicating the fate of cyanide in mammals has been designed by Williams (13) as follows:



In *S. granarius* CN^- apparently goes into the CN^- pool and subsequently may follow at least four pathways, i.e. to cyanocobalamin, to carbon dioxide, a small amount may be expired as HCN (although this could not be demonstrated), and a major portion was excreted in the faeces as amino acids and

other compounds. The finding that the cyanide carbon was incorporated in the aspartic acid part of a polypeptide and that this carbon occurred in the carboxyl group of the aspartic acid may provide a clue to the pathway of cyanide excretion in this insect.

Investigation of the end products of metabolism revealed the presence of a considerable number of amino acids and other compounds in the faeces of *S. granarius*. Usually it is believed that only in Homopterous insects are amino acids excreted in large amounts (14). It is possible that many of the compounds demonstrated here are not end products of metabolism but rather come from unabsorbed food. However, the detection of labelled glycine and aspartic acid in the faeces indicated that they were metabolic products. These insects, then, were not only able to excrete amino acids, but they were also able to synthesize them and furthermore this synthesis was involved in the elimination of cyanide from them.

All of these observations serve to demonstrate the complexity of the action of cyanide on *S. granarius* and to indicate that its detoxication and elimination takes place by previously unknown pathways.

Acknowledgments

It is a pleasure to acknowledge the many kindnesses shown to me by members of the staff at the Imperial College Field Station, Sunninghill, Berks. I am particularly indebted to Dr. A. B. P. Page and Dr. F. Call, who gave many helpful suggestions and encouragement. Dr. J. R. Robinson of the Canada Department of Agriculture, London, Ontario, kindly assisted me with some of the isotope work and Dr. E. Y. Spencer and Dr. W. Chefurka gave helpful suggestions for improvement of the manuscript.

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THE INVOLVEMENT OF CARBON DIOXIDE IN THE TOXICITY OF OXYGEN AT HIGH PRESSURE[1]

I. G. WALKER

With the technical assistance of W. J. WATSON

Abstract

Mice were adapted to living in an environment containing high concentrations of carbon dioxide by exposing them to an atmosphere in which the concentration of this gas was increased each day by 2% to a maximum of 10% or 20%. The animals were then exposed to an atmosphere of 100% oxygen at 75 p.s.i. (gauge). The time of onset of convulsions under these conditions was significantly greater than that in untreated mice. This result in conjunction with the findings of others was interpreted to mean that animals exposed to oxygen at high pressure suffer some degree of impairment to carbon dioxide transport which contributes to the production of symptoms.

Introduction

When animals are exposed to oxygen at high pressure (OHP) the hemoglobin remains fully or nearly fully saturated and sufficient oxygen to meet the demands of the tissues is carried in physical solution by the plasma. The normal transport of carbon dioxide by the blood depends largely on the formation of reduced hemoglobin. For this reason, Gesell, in 1923 (1), proposed that autointoxication with carbon dioxide was an important contributing cause of oxygen convulsions. Two kinds of evidence have been proposed to support this hypothesis. The first is that small amounts of carbon dioxide in the oxygen at high pressure induce convulsions much faster than pure oxygen at high pressure (2, 3, 4). Lambertsen *et al.* (5) do not consider that this evidence supports Gesell's hypothesis. They demonstrated that the inhaled carbon dioxide produces an arterial hypercapnea which causes a cerebral vasodilatation and permits a larger amount of oxygen to reach the brain tissue. The second kind of evidence has been provided by measurements of tissue carbon dioxide tension (p_{CO_2}) in animals exposed to OHP; Campbell (6) and Taylor (4) sampled the composition of a subcutaneous gas bubble and Seelkopf and Von Wertz (7) sampled a fluid depot. All of these workers reported a rise in the depot p_{CO_2} that preceded the onset of convulsions. Lambertsen *et al.* (8) pointed out errors in the technique used by these authors and repeated the work using methods which obviated the errors. The reinvestigation showed that the p_{CO_2} in the depot rose only when the animals were in a terminal state and were obviously suffering from respiratory distress.

[1]Manuscript received July 14, 1961.
Contribution from the Biochemistry Section, Defence Research Medical Laboratories, Toronto, Ontario. This is DRML Report No. 245-1.

Bahnson and Mathews (9) have also used the gas bubble technique in much the same way as the Lambertsen group. Their results showed a definite pre-convulsive rise in depot p_{CO_2} in contrast to the finding of Lambertsen *et al.* Measurements of pH, p_{CO_2}, and the carbon dioxide content of blood have been made on dogs by Behnke *et al.* (10) and on men by Lambertsen *et al.* (11) during exposure to OHP. These authors concluded that their findings did not support the theory of carbon dioxide intoxication. Both groups of authors, however, reported a significant elevation of venous p_{CO_2} which suggests that there was some degree of impairment in carbon dioxide transport. In 1958, Van de Berg (12) found that fluoroacetate-induced accumulation of citric acid in the rat was reduced by exposure to OHP, or by the administration of a carbonic anhydrase inhibitor (acetazoleamide) or of an acidifying agent (ammonium chloride). Acetazoleamide and ammonium chloride both affect carbon dioxide metabolism. Van de Berg's results suggested again the possibility that carbon dioxide was implicated in the production of symptoms by OHP. The rationale of the work to be reported is that, if the production of convulsions by OHP is partly due to an impairment in carbon dioxide transport, then an animal that is tolerant to high concentrations of carbon dioxide should be more resistant to the convulsant effects of OHP. The present findings appear to substantiate this hypothesis.

Methods

Adaptation to Carbon Dioxide

Young white mice of the Connaught strain weighing 22–28 g were adapted in a wooden box measuring 38 cm on each side; this contained a small circulating fan and a removable front panel of lucite. The mice were placed on a coarse wire screen over a tray containing sawdust. No more than 20 mice were put in the chamber. Purina fox chow held in a coarse mesh container, and water, were supplied ad libitum. Air was supplied from a compressed air line and carbon dioxide from a cylinder. Each gas line was fitted with a reducing valve and a flow meter. The lines were joined through a Y-connection before passing into the chamber near the top and near the bottom. The flow rate of air was maintained at 3 liters per minute and the flow rate of carbon dioxide was adjusted to give the desired carbon dioxide concentration within the chamber. The carbon dioxide concentration was raised 2% each day and it was measured at the top and bottom of the chamber by the technique of Fry (13). The chamber was opened once a day to clean the refuse tray, and to supply food and water. The animals were weighed quickly and returned to the chamber. The approximate amount of food consumed was estimated by weighing the pellets remaining in the food container.

The sham-adapted animals were kept in an identical box with air at 3 liters per minute, but no carbon dioxide, flowing into it. The amount of food supplied to these animals was the same as had been consumed on the previous day by the group exposed to carbon dioxide.

The mice undergoing adaptation to carbon dioxide appeared to be as active as the untreated or sham-adapted mice. There was no doubt that they were able to tolerate a higher carbon dioxide concentration than untreated mice. When mice were exposed, without prior adaptation, to an atmosphere containing 20% carbon dioxide, they soon became prostrate. They retained consciousness over the hour that they were observed but did not resist attack by adapted mice.

Exposure to Oxygen

The exposure chamber* was a steel cylinder having an internal diameter 12 in. and length 23 in. A fan outside the chamber circulated the gas and passed it through a carbon dioxide absorber. There were heavy glass ports in the ends of the chamber to provide illumination and to allow viewing of the animals. Oxygen was supplied from a cylinder.

The mice were placed in a lucite box which contained holes to allow good gas circulation. The box was partitioned so that the mice were separated. Wire cages were found unsuitable because the mice gripped the cage with feet and teeth and successfully prolonged the time before they convulsed. Ten mice in two boxes could be exposed at one time and untreated, adapted, and sham-adapted mice were always exposed simultaneously. After the mice were placed in the chamber, it was first flushed well with oxygen and then the pressure was raised to 75 p.s.i. (gauge) over a period of 2 minutes. Timing began when the final pressure had been attained. A convulsion was scored when a generalized spasm occurred. Measurements of the gas composition in the chamber were made on samples taken in the vicinity of the mice. The oxygen concentration was at least 99% and the carbon dioxide concentration was below the sensitivity (about 1%) of the method.

Results

The results of all convulsion tests are given in Table I. The mean time to the onset of convulsions (convulsion time) for untreated mice exposed to 100% oxygen at 75 p.s.i. (gauge) did not differ significantly between experiments so all the values were pooled. The values from the sham-adapted groups were pooled for the same reason.

Experiments 1 and 2 demonstrate that the convulsion time can be increased significantly by a prior adaptation to 10% carbon dioxide. To achieve this effect with 10% carbon dioxide a necessary condition would appear to be, that, once the final concentration of carbon dioxide has been attained, the mice remain in this environment for several days (experiment 3). Experiments 4 to 6 show that a significantly increased convulsion time can be achieved by a prior adaptation to 20% carbon dioxide. However, as seen in experiments 6 to 8, if the adaptation to 20% carbon dioxide extends over about 10 days, or

*The chamber was designed by Dr. C. A. de Candole and was built under the supervision of the Technical Services Group of the Defence Research Medical Laboratories. Further details will be sent on request.

TABLE I

The time to convulsion of mice in 100% O_2 at 75 p.s.i. (gauge) after prior adaptation to an environment containing a high concentration of CO_2

Expt. No.	Comment	Mean time to convulsion, minutes ±s.d. (no. of mice)		Significance of the difference between experimental and untreated mice*
	Untreated mice from all experiments	8.3± 5.6	(65)	
	Sham-adapted mice from all experiments except No. 9	10.3± 5.2	(74)	$0.05 < P < 0.01$
1	Adapted to 10% CO_2 over 5 days, remained at 10% CO_2 until 9th day	21.4±10.4	(9)	$P < 0.001$
2	Adapted to 10% CO_2 over 5 days, remained at 10% CO_2 until 17th day	19.2± 7.7	(11)	$P < 0.001$
3	Adapted to 10% CO_2 over 5 days	13.9± 4.5	(6)	Not significant
4	Adapted to 20% CO_2 over 10 days	26.5± 7.0	(10)	$P < 0.001$
5	Adapted to 20% CO_2 over 11 days	17.7± 9.3	(10)	$P < 0.001$
6	Adapted to 20% CO_2 over 13 days	17.7±13.2	(5)	$P < 0.01$
7	Adapted to 20% CO_2 over 14 days	12.5± 2.4	(5)	Not significant
8	Adapted to 20% CO_2 over 10 days, remained at 20% CO_2 until 13th day	8.0± 6.1	(6)	Not significant
9	CO_2 content of sham-adapting chamber maintained below detectable level. Treatment lasted 11 days	7.7± 2.5	(10)	Not significant

*"Student's" *t*-test.

if the animals are kept in 20% carbon dioxide for several days, the convulsion time decreases. This decreased resistance to OHP may be related to debilitation caused by the prolonged stay in the high concentrations of carbon dioxide.

The effect on convulsion time of adaptation to a carbon dioxide concentration lower than 10% was not studied. Adaptation to lower concentrations of carbon dioxide might be effective in increasing the convulsion time. It will be noted that the convulsion time of the sham-adapted mice is slightly greater than that of the untreated mice and the difference may be significant. A possible explanation is that the carbon dioxide concentration at the bottom of the sham-adapting chamber was often about 1% and exposure to this atmosphere may have been sufficient to have evoked slightly increased convulsion times. The 1% level of carbon dioxide was presumably due to inefficient removal of respiratory carbon dioxide. In experiment 9, the carbon dioxide in the adapting chamber was maintained below a detectable level by increased air circulation and by the inclusion of a carbon dioxide absorbent in the chamber. The convulsion time in OHP for the mice treated in this fashion was the same as that of untreated mice.

The food consumption of the mice undergoing adaptation to carbon dioxide decreased and the mice lost weight. In experiment 4, the average weights at the beginning and end of the experiment were 27 g and 24 g respectively. In experiment 5 the average weight remained at 23 g to 24 g. Because of the restricted food allowance the changes in weight of the sham groups were identical with those in the treated groups. Untreated animals of this age gained about 0.5 g per day.

Discussion

Gilbert *et al.* (14) have demonstrated that previous fasting of mice increased their survival time when they were exposed to 6 atmospheres of oxygen. In the present study the marked difference in convulsion times between adapted mice and sham-adapted mice, both of which exhibited identical patterns in weight change, suggests that the elevated convulsion time of adapted mice was not due to a nutritional cause.

In analyzing the present results, the assumed premise is that during exposure to OHP, carbon dioxide transport is not normal and that this condition can lead to changes in tissue metabolism. In the experiments of Lambertsen *et al.* (11), in which men were exposed to 3.5 atmospheres (gauge pressure) of pure oxygen, the venous p_{CO_2} was increased by 5–6 mm Hg and the arterial p_{CO_2} was decreased by about the same amount due to hyperventilation. The arteriovenous difference in volumes per cent carbon dioxide was not altered and one must agree with Lambertsen *et al.* that there is no autointoxication with carbon dioxide in the sense that the normal amount of this gas is being transported from tissues to lungs. However, the elevation of venous p_{CO_2} can be regarded as a disturbance in carbon dioxide transport and the hyperventilation a compensatory response. Armstrong *et al.* (15) have recently presented convincing evidence for a chemoreceptor that responds to physicochemical changes

in mixed venous blood and mediates changes in ventilation. The proposal that OHP alters carbon dioxide transport by maintaining hemoglobin in the oxygenated state, fits well with this concept. That is, if the hemoglobin system were not functioning, one would predict a rise in venous p_{CO_2} followed by hyperventilation. Also, if OHP does not lead to an alteration in carbon dioxide transport, one must conclude that hemoglobin is not necessary for the transport of this gas. The work of Van de Berg (12) on the administration of acetazolamide or ammonium chloride is an example of the effect of altered carbon dioxide transport on tissue metabolism. He observed the same effect on tissue metabolism when he exposed animals to OHP.

In the present experiments, mice became acclimatized to an atmosphere high in carbon dioxide. Presumably some physiological mechanism was modified to meet this requirement. At the same time the mice developed a greater tolerance for OHP. It is supposed that the physiological modification which allows a greater tolerance to carbon dioxide is also the one which provides an increased tolerance to OHP. Continued exposure to high levels of carbon dioxide may induce a general adaptation, perhaps quite unconnected to carbon dioxide transport, which protects against high concentrations of carbon dioxide and oxygen alike. A change in excitability of the central nervous system would be such a possibility. Information on this point is not available and from the evidence cited above it is simpler to assume that animals undergoing exposure to OHP suffer some impairment of carbon dioxide transport which contributes to the production of convulsions.

Conclusion

1. Mice adapted to an atmosphere high in carbon dioxide (10 or 20%) are more resistant to the convulsant effects of oxygen at 6 atmospheres pressure than are untreated mice.

2. The increased tolerance to oxygen at high pressure may result from an increased ability to withstand the effects of interference with the transport of carbon dioxide.

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THE EFFECTS OF LEAD AND TIN ORGANOMETALLIC COMPOUNDS ON THE METABOLISM OF RAT BRAIN CORTEX SLICES[1]

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Abstract

The effects of tetraethyl lead, tetraethyl tin, triethyl lead, and triethyl tin on the metabolism of rat brain cortex slices have been studied. Tetraethyl lead and tetraethyl tin inhibit the active transport of amino acids into rat brain cortex slices at concentrations and under conditions that show no effect on the glucose metabolism of the slices. Tetraethyl lead and tetraethyl tin inhibit the oxidation of L-glutamate by rat brain slices. This effect can be accounted for on the basis of the inhibitory action of these two substances on the transport of the amino acid into the brain tissue.

Tetraethyl lead and tetraethyl tin abolish, at low concentrations, potassium-stimulated brain slice respiration in presence of glucose, having little or no effect on unstimulated brain slice respiration. However, the respiration of rat brain cortex slices previously treated with phospholipase A is highly sensitive to tetraethyl lead.

The inhibitory effects of the two tetraethyl compounds show differences from those of their triethyl derivatives indicating that the effects of the former substances are not due to admixture with, or conversion to, the latter substances.

The brain slices of rats poisoned with either tetraethyl lead or tetraethyl tin are unable to effect the active transport of amino acids. The appearance of this biochemical abnormality coincides with the manifestation of neuropathological symptoms.

The mode of action of tetraethyl lead and of tetraethyl tin on brain metabolism in vitro is discussed. It is suggested that they may act on phospholipid groups concerned with amino acid and cation transport at the cell membrane.

Introduction

The fact that 4EL* poisoning disturbs the normal function of the central nervous system is now well established. The development of acute mental symptoms is probably its most obvious manifestation and the one that carries great importance for prognosis. Histopathological changes have been found in the brains of man and of laboratory animals following 4EL poisoning (1, 2, 3).

Cremer (4, 5) has advanced the view that the toxicity in vivo of 4EL or of 4ET is due to the formation of 3EL or of 3ET, respectively, by an enzymatic deethylation system found in liver tissue. The triethyl derivatives affect metabolic reactions in the central nervous system, both 3EL and 3ET being potent uncouplers of oxidative phosphorylation (4, 6, 7).

Experimental findings, which will be presented in this paper, indicate that 4EL and 4ET can directly affect metabolism in the central nervous system.

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*The following abbreviations are used: 4EL, tetraethyl lead; 4ET, tetraethyl tin; 3EL, triethyl lead; 3ET, triethyl tin; TCA, trichloracetic acid; DNP, 2,4-dinitrophenol.

Materials and Methods

Materials

All amino acids used were obtained from the Nutritional Biochemicals Corporation. Radioactive substances were bought from Merck Co. Ltd., and the Atomic Energy Commission of Canada. 4EL was a generous gift of the DuPont Company of Canada Ltd. 4ET was obtained from Brickman and Co., Montreal. 3EL and 3ET were kindly sent to us by Dr. W. N. Aldridge of the Medical Research Council Laboratories, Surrey, England.

Tetraethyl lead and tetraethyl tin were dissolved in *n*-propanol and stored at 0°. Prior to each experiment an appropriate volume was diluted with distilled water and added to the experimental vessels so that the final concentration of propanol never exceeded 0.3%. Control experiments were systematically carried out to ensure that this quantity of propanol had little or no effect on the various aspects of brain metabolism in vitro that were studied.

Tissue Preparations

Tissues from hooded adult male rats bred in this Institute, and weighing about 150 g, were used. Tissue slices were prepared with the Stadie–Riggs slicer. Brain homogenates were prepared by treatment of whole cerebral hemispheres suspended in the Ringer medium described below in a Potter–Elvehjem glass homogenizer at 0° C for approximately 1 minute.

Incubation

A Krebs–Ringer incubation medium of the following composition was used for tissue slices and homogenates: NaCl 128 mmolar, KCl 5 mmolar, $CaCl_2$ 3.6 mmolar, $MgSO_4$ 1.3 mmolar, KH_2PO_4–K_2HPO_4 1.3 mmolar (pH 7.4).

Tissue preparations were incubated in the conventional Warburg apparatus at 37° C. Radioactive substrates were usually tipped into the main compartment of the vessels at zero time. Aerobic incubations were carried out in an atmosphere of oxygen with KOH in the center wells to absorb CO_2. Pure nitrogen was used as the gas phase in anaerobic experiments. The total volume of the incubation medium was 3 ml.

Assay of Radioactive CO_2

At the end of incubation, 0.3 ml of 30% TCA was tipped into the main compartment of the vessels to stop all metabolic processes and liberate any bound CO_2. Incubation was continued for an additional 30 minutes after which time the contents of the center wells were quantitatively transferred to test tubes containing $NaHCO_3$ carrier (1 ml of 1.2% $NaHCO_3$). CO_2 was precipitated as $BaCO_3$ by the addition of two drops of 2 *M* NH_4Cl and excess $BaCl_2$. The precipitate was washed several times with water and finally with acetone. An aliquot of the final acetone suspension was then plated on aluminum foil plates and counted under a mica window Geiger–Müller tube connected to a Tracerlab Utility Interpolation scaler. A correction for self-absorption was applied, using a self-absorption curve, constructed by the method of Calvin

et al. (8) for our counting conditions. The experimental variation in the results recorded did not exceed ±15%.

Measurements of Uptake of Radioactivity

Tissue slices were removed from the incubation medium after a specified time and rinsed for 2–3 seconds in ice-cold water. They were then dropped into 3 ml 80% ethanol, homogenized, and allowed to stand at room temperature for 90 minutes to ensure complete extraction of free amino acids. After centrifugation an aliquot of the supernatant was plated and counted. The experimental variations in the results recorded did not exceed ±15%.

Chromatography and Radioautography

The 80% ethanol extract was subjected to two-dimensional paper chromatography on Whatman No. 1 filter paper, 10 inches square. The following solvents were used:

phase I: secondary butanol – 90% formic acid – water (70:11:17);

phase II: phenol – water – ammonia (422:110:1.7).

The chromatograms were developed in each phase for 18 hours. Radioactive spots were located by direct contact of the chromatograms with a Kodak, No-Screen, blue base, X-ray plate. Radioactivity measurements were made on paper by counting with a mica window Geiger-Müller probe. After counting, all ninhydrin-positive spots were located by dipping the chromatogram into a solution of 0.04% ninhydrin in acetone and heating at 100° C for 3 minutes. Radioactive amino acids were identified by the ninhydrin reaction and by comparison with the appropriate standards.

Results

The Effects of Tetraethyl Lead on Some Metabolic Activities of Brain Slices

It has been claimed (4) that 4EL does not disturb the normal respiratory metabolism of brain slices when it is added in vitro. Our experiments (Table I) show that although this claim is true as far as glucose metabolism is concerned

TABLE I

The effects of tetraethyl lead (4EL) on some metabolic activities of rat brain cortex slices

Additions	Control			With 8.1×10^{-4} *M* 4EL		
	Q_{O_2}	Uptake	$C^{14}O_2$	Q_{O_2}	Uptake	$C^{14}O_2$
20 mmolar DL-glutamate-1-C^{14}	12.8	219	405	9.1	142	295
20 mmolar DL-glutamate-1-C^{14} 10 mmolar glucose	13.9	446	326	7.6	188	136
10 mmolar glucose-U-C^{14}	11.9	216	182	12.0	207	176
10 mmolar glucose-U-C^{14} 10 mmolar L-glutamate	14.0	246	132	8.1	214	146

NOTE: Incubation: 60 minutes; gas phase, oxygen.
Uptake: Figures give total radioactivities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. substrate introduced per vessel.
$C^{14}O_2$: Figures give total radioactivities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. substrate introduced per vessel.

it does not apply to the metabolism of glutamic acid. 4EL, at a concentration of 8.1×10^{-4} *M*, inhibits the oxygen uptake of brain slices when glutamic acid is the sole substrate. This inhibition of oxygen uptake is even greater when glucose as well as glutamic acid is present. The effect is accompanied by an inhibition of the $C^{14}O_2$ evolution from glutamate-1-C^{14}. This concentration of 4EL also inhibits, almost completely, the glucose-dependent accumulation of intracellular radioactivity upon incubation with glutamate-1-C^{14}.

Glutamic Acid "Uptake", Glutamine Formation, and the Effects of 4ET

It is well known (9) that brain cortex slices, incubated in the presence of glucose, accumulate glutamic acid from the medium against a diffusion gradient. Accordingly, experiments were carried out to observe the effects of 4EL on the uptake of glutamate by brain cortex slices. Glutamate-1-C^{14} was used as substrate. It was considered desirable to ascertain whether all the activity measured in the 80% ethanol extract of the slices represents radioactive glutamic acid or a mixture of glutamate and other metabolites derived from it. To accomplish this, the extracts were subjected to two-dimensional paper chromatography followed by radioautography. The only radioactive substance that could be found in the extract, other than glutamic acid, was glutamine and its radioactivity was about 1/30th that of glutamate both in the absence and in the presence of glucose (see Table II).

TABLE II

Radioactive amino acid pattern of rat brain cortex slices after incubation with glutamate-1-C^{14} with and without tetraethyl tin

Radioactive amino acids formed	Additions			
	Nil	10 mmolar glucose	10^{-4} *M* 4ET	10 mmolar glucose $+10^{-4}$ *M* 4ET
Glutamic acid	12,680	29,110	7,460	4,260
Glutamine	436	911	209	No activity detected

NOTE: Incubation: 60 minutes; gas phase, oxygen. All vessels contained 4 mmolar DL-glutamate-1-C^{14} in a final volume of 1 ml.
Values given are activities of radioactive amino acids expressed as c.p.m. per 100 mg wet weight of tissue per 10^6 c.p.m. introduced, as counted on Whatman No. 1 filter paper after chromatography.
All figures are averages of results of at least two experiments.

Results given in Table II also demonstrate the suppressing action of 4ET, resembling that of 4EL, on the energy-dependent transport of glutamate and on glutamine formation.

An investigation was then undertaken of the time course of glutamate-1-C^{14} accumulation in rat brain cortex slices in the presence or absence of glucose, 4EL, and a typical uncoupler of oxidative phosphorylation such as DNP, under aerobic or anaerobic conditions. The results obtained are presented in Table III. It will be seen that a level of radioactive intracellular glutamate of approximately 250 c.p.m. per mg dry weight of tissue (per 10^5 c.p.m. glutamate-1-C^{14} introduced) is obtained in the absence of glucose. Accumula-

TABLE III

The rate of uptake of glutamate-1-C^{14} by rat brain cortex slices

Incubation time (minutes)	Total radioactivity of 80% ethanol extract with additions of: Control	10^{-4} *M* 4EL	10 mmolar glucose	10 mmolar glucose $+10^{-4}$ *M* 4 EL	10 mmolar glucose $+7.5\times10^{-5}$ *M* DNP
			Aerobic incubation		
2	24	21	27	18	17
15	169	141	165	160	146
30	236	175	336	190	165
45	245	168	412	224	183
60	236	161	440	246	185
75	—	157	410	231	—
90	—	168	389	248	—

Incubation time (minutes)	Total radioactivity of 80% ethanol extract with additions of: Control	10 mmolar glucose	8.1×10^{-4} *M* 4EL	10 mmolar glucose $+8.1\times10^{-4}$ *M* 4EL
		Anaerobic incubation		
60	275	292	242	278

NOTE: Incubation: (*a*) Aerobic; gas phase, oxygen. (*b*) Anaerobic; gas phase, pure nitrogen. All vessels contained 20 mmolar DL-glutamate-1-C^{14}.
All figures given are total radioactivities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^{-5} c.p.m. introduced per vessel.
Figures under "Aerobic incubation" and "Anaerobic incubation" are averages of results of three and two experiments respectively.

tion of glutamate above this level cannot be detected under anaerobic conditions in the presence or in the absence of glucose. In the presence of glucose, aerobic accumulation of glutamate proceeds at a fairly constant rate up to about 60 minutes after the start of incubation. In the next 30-minute interval, no additional accumulation can be detected and, in fact, there is a gradual fall in the level of intracellular glutamate. The presence of 4EL (10^{-4} *M*) abolishes the glucose-dependent active uptake of glutamate almost completely, resembling in this respect the effect of 7.5×10^{-5} *M* DNP, which uncouples oxidative phosphorylation in brain slices with no inhibition of respiration (10). There is no effect of 4EL on the anaerobic uptake of glutamate-1-C^{14}.

The Effect of 4EL on the Uptake of Amino Acids Other than Glutamic Acid

A series of experiments was carried out to determine whether the effects of 4EL on the uptake of glutamic acid are specific for this amino acid. The results (Table IV) show that the transport of glycine, serine, or alanine into brain cortex slices is increased considerably by the presence of glucose and that 10^{-4} *M* 4EL inhibits the glucose-dependent active uptake of these amino acids by brain cortex slices. The accumulation of glycine in slices of rat brain cortex has been previously demonstrated (11). With alanine-1-C^{14} and serine-1-C^{14} all the radioactivity of the ethanol extracts is due to these two amino acids present as such. No other radioactive spot can be detected after chromatography of these extracts. With methionine-S^{35}, the presence of glucose does not

TABLE IV

The effects of tetraethyl lead (4EL) on amino acid uptake by rat brain cortex slices

Additions	Uptake	
	Control	10^{-4} *M* 4EL
2 mmolar glycine-1-C^{14}	680	430
2 mmolar glycine-1-C^{14} + 10 mmolar glucose	1625	526
4 mmolar DL-methionine-S^{35}	334	275
4 mmolar DL-methionine-S^{35} + 10 mmolar glucose	337	286
4 mmolar DL-alanine-1-C^{14}	408	297
4 mmolar DL-alanine-1-C^{14} + 10 mmolar glucose	830	334
4 mmolar DL-serine-1-C^{14}	590	—
4 mmolar DL-serine-1-C^{14} + 10 mmolar glucose	814	266

NOTE: Incubation: 60 minutes; gas phase, oxygen.
Uptake: Figures given are total radioactivities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^{-6} c.p.m. introduced.
All figures are the averages of results of three experiments, except those with serine-1-C^{14} which are the averages of results of two experiments.

increase the level of intracellular radioactivity and in this case 4EL (10^{-4} *M*) has little, if any, effect on the transport.

Specificity of Action of 4EL

Results given in Table V show that the effects of 4EL on glutamate accumulation in rat brain cortex slices in vitro do not occur with rat kidney cortex slices or with liver slices. Undoubtedly this is connected with the fact that 4EL abolishes the active, or glucose-dependent, transport of the amino acid, a process much more evident in the brain than in kidney cortex or liver.

TABLE V

The effect of 4EL on amino acid uptake in slices of different tissues of the rat

Tissue	Additions	Total activity of 80% ethanol extracts with addition of 4EL at molar concn. of:			
		Control	6.5×10^{-5}	2.7×10^{-4}	8.1×10^{-4}
Brain cortex	20 mmolar DL-glutamate-1-C^{14} 10 mmolar glucose	431	206	211	192
Kidney cortex	20 mmolar DL-glutamate-1-C^{14}	141	—	—	128
	20 mmolar DL-glutamate-1-C^{14} 10 mmolar glucose	156	—	—	154
Liver	20 mmolar DL-glutamate-1-C^{14} 10 mmolar glucose	84	—	—	92

NOTE: Incubation: 60 minutes; gas phase, oxygen.
Figures are total activities of 80% ethanol extract in c.p.m. per mg dry weight of tissue per 10^{6} c.p.m. introduced.
All figures are averages of results of three experiments.

Amino Acid Formation from Glucose in Presence of Rat Brain Cortex Slices and Effects of 4EL

Radioactive glucose is converted into radioactive amino acids by brain cortex slices incubated aerobically in an appropriate Ringer medium. It has already

been shown that the pattern of amino acids derived from glucose is greatly affected by substances disturbing carbohydrate metabolism in brain (12). Substances that have uncoupling effects diminish the synthesis of labelled glutamine, a process that is adenosine triphosphate dependent. Results given in Table VI show that 10^{-4} *M* 4EL has but little effect on the relative yields of radioactive amino acids from radioactive glucose, a finding consistent with the lack of effect of this concentration of 4EL on glucose metabolism under the given experimental conditions.

TABLE VI

Radioactive amino acid patterns of rat brain cortex slices after incubation with glucose-U-C^{14} and effects of 4EL (10^{-4} *M*)

Radioactive amino acid	Radioactivity of amino acid spots with additions of:	
	Nil	10^{-4} *M* 4EL
Glutamic acid	4620	4345
Aspartic acid	1860	1910
Glutamine	1955	1750
γ-Aminobutyric acid	735	685
Alanine	680	635

NOTE: Incubation: 60 minutes; gas phase, oxygen. All vessels contained 5 mmolar glucose-U-C^{14} in a final volume of 1 ml.
Figures are activities of radioactive amino acids expressed as c.p.m. per 100 mg wet weight of tissue per 10^6 c.p.m. introduced, as counted on Whatman No. 1 filter paper after chromatography. Control figures are averages of results of six experiments; all others are the averages of results of three experiments. Mean deviation = ±7%.

The Effects of Tetraethyl Tin (4ET) on the Metabolism of Rat Brain Cortex Slices

The effects of 4ET on some aspects of the metabolism of brain cortex slices are shown in Tables VII and VIII. It will be seen that the results on the uptake of the amino acids are similar to those obtained with 4EL. 4ET, at a concentration of 10^{-4} *M*, does not interfere with the rate of oxygen consumption, or of $C^{14}O_2$ production, when glucose-U-C^{14} is the substrate. The same concentration of 4ET inhibits the active uptake of glutamate and glycine.

It is interesting to observe from the results given in Table II that the glucose-stimulated transport of glutamate into rat brain cortex slices is not only blocked by the presence of 0.1 mmolar 4ET, but that the drug brings about (in presence of glucose) a reduction in the levels of radioactive glutamate and glutamine below those found in the absence of glucose. Probably this is connected with the fact that the largest diminution in the rate of respiration, accomplished by 4ET or 4EL at 0.1 mmolar, occurs when glutamate is present together with glucose.

The Effects of Tetraethyl Lead and Tetraethyl Tin on Potassium-Stimulated Brain Respiration

It is noteworthy that one of the effects of 4EL and 4ET is the large inhibition of the oxygen consumption of brain slices when both glutamate and glucose are present (Tables I and VII). This inhibition is considerably larger than that

TABLE VII

The effects of tetraethyl tin (4ET) on some metabolic activities of rat brain cortex slices

Additions	Q_{O_2}			Uptake			$C^{14}O_2$		
	4ET added			4ET added			4ET added		
	Nil	10^{-4} *M*	3×10^{-5} *M*	Nil	10^{-4} *M*	3×10^{-5} *M*	Nil	10^{-4} *M*	3×10^{-5} *M*
20 mmolar DL-glutamate-1-C^{14} 10 mmolar glucose	13.4	7.8	12.2	420	186	300	292	186	252
10 mmolar glucose-U-C^{14}	11.2	11.2	12.2	—	—	—	173	182	185
2 mmolar glycine-1-C^{14} 10 mmolar glucose	10.9	11.8	12.4	1410	418	—	—	—	—

NOTE: Incubation: 60 minutes; gas phase, oxygen.
Uptake: Figures are total radioactivities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. substrate introduced per vessel.
$C^{14}O_2$: Figures are total activities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^5 substrate introduced per vessel.
All figures are averages of results of three experiments.

obtained in the presence of either of the substrates alone. It is known (13) that the presence of both glucose and glutamate is necessary for the prevention of loss of potassium ions from brain slices into the incubation medium. Thus, when both glucose and glutamate are present the slices metabolize under conditions of relatively high intracellular potassium. A series of experiments was undertaken, therefore, to study the effects of 4EL or 4ET on potassium-stimulated brain respiration.

TABLE VIII

The effects of tetraethyl lead (4EL) and tetraethyl tin (4ET) on potassium-stimulated metabolism of rat brain cortex slices

Additions			Additions		
			10 mmolar glucose-U-C^{14}	20 mmolar DL-glutamate-1-C^{14}	20 mmolar DL-glutamate-1-C^{14} +10 mmolar glucose
5 mmolar KCl	Control	Q_{O_2}	10.9	12.3	13.9
		Uptake	241	284	488
		$C^{14}O_2$	175	387	332
	10^{-4} *M* 4EL	Q_{O_2}	11.7	9.5	7.5
		Uptake	232	206	216
		$C^{14}O_2$	181	284	168
	10^{-4} *M* 4ET	Q_{O_2}	12.3	—	8.2
		Uptake	184	—	192
		$C^{14}O_2$	190	—	198
105 mmolar KCl	Control	Q_{O_2}	18.5	13.1	16.7
		Uptake	308	158	232
		$C^{14}O_2$	330	520	353
	10^{-4} *M* 4EL	Q_{O_2}	8.9	8.0	8.2
		Uptake	196	183	221
		$C^{14}O_2$	155	388	238
	10^{-4} *M* 4ET	Q_{O_2}	4.6	—	4.8
		Uptake	118	—	212
		$C^{14}O_2$	93	—	202

NOTE: Incubation: 60 minutes; gas phase, oxygen. In the vessels containing 105 mmolar KCl 0.3 ml of 1 *M* KCl was tipped in the main compartment of the vessels together with the radioactive substances at zero time.
Uptake: Figures shown are total radioactivities of 80% ethanol extracts in c.p.m. per mg dry weight oft issue per 10^5 c.p.m. introduced.
$C^{14}O_2$: Figures are total activities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. introduced.
All figures are averages of results of three experiments.

The results, given in Table VIII, show that at 10^{-4} *M* either 4EL or 4ET inhibits the potassium-stimulated rate of respiration, as shown by reduction of oxygen consumption values and of $C^{14}O_2$ evolved from glucose-U-C^{14}. It has already been shown that the same concentration of either substance does not interfere with the unstimulated respiration when glucose is the only substrate. This result can be explained if 4EL or 4ET interferes with cation transport at the neuronal membrane, thus nullifying the effects of added potassium ions. It may be accounted for equally well if the drug inhibits mitochondrial DPNH oxidation and the coupled synthesis of adenosine triphosphate. However,

results show that brain mitochondrial respiration is relatively insensitive to 4EL.

Another effect clearly shown by the results in Table VIII is that, in the presence of high concentrations of KCl, the energy-dependent accumulation of glutamic acid in the slices is reduced. It may be noted that it is already known (11) that glycine incorporation into brain proteins is suppressed by high concentrations of KCl.

The Effects of 3EL and 3ET on the Metabolism of Rat Brain Cortex Slices

The high toxicities of 3EL and 3ET, reported by Aldridge and Cremer (4, 6, 7), made it necessary to investigate the possibility that the effects obtained in vitro with 4EL and 4ET may be due to the presence of small quantities of the triethyl derivatives of lead and tin in the samples of the tetraethyl compounds used. An investigation was, therefore, made of the effects of triethyl lead and triethyl tin at small concentrations and also of mixtures of the triethyl and tetraethyl compounds on the oxidation of glucose and glutamate and on the uptake of glutamate by brain slices.

The results, reported in Tables IX and X, lead to the following conclusions:

(*a*) A large inhibition of the rates of oxygen uptake and of $C^{14}O_2$ production, with glucose-U-C^{14} as the substrate, takes place with concentrations of the triethyl compounds between 10^{-4} *M* and 10^{-6} *M* (Table IX).

(*b*) No inhibition of glutamate metabolism larger than, or even equal to, that of glucose metabolism takes place at any concentration of the triethyl compounds used. The inhibition of glutamic acid uptake only occurs when there is an inhibition of all metabolic processes investigated.

(*c*) With mixtures of tetraethyl (10^{-4} *M*) and triethyl (10^{-6} *M*) compounds (Table X), the depression of Q_{O_2} and $C^{14}O_2$ output, with glucose-U-C^{14} as substrate, is similar to that obtained in the presence of 10^{-6} *M* 3EL or 3ET alone (Table IX). Moreover, the specific inhibition of glutamate oxidation and uptake effected by the tetraethyl compounds occurs equally well when the triethyl compounds are also present.

These results lead to the conclusion that the effects in vitro of 4EL or 4ET, at the concentrations tested, are not due to admixture with, or formation of, small quantities of 3EL or 3ET. The former substances although showing definite effects on amino acid metabolism show none on glucose breakdown, while the latter, at concentrations affecting amino acid metabolism, also affect glucose metabolism.

Effects of 4EL on Rat Brain Cortex Slices in Presence of Phospholipase A

In order to throw more light on the mode of action of 4EL its effects on rat brain cortex slices treated with phospholipase A (heated snake venom) were studied. Phospholipase A was prepared from cobra venom as previously described (14). It has been shown that, with phospholipase A, the brain cell membranes are modified and the altered respiratory metabolism of brain cortex slices leads to a fall in P/O ratio followed by a decrease in respiration (14).

TABLE IX

The effect of triethyl lead (3EL) chloride and triethyl tin (3ET) sulphate on some metabolic activities of rat brain cortex slices

Additions			Additions: 10 mmolar glucose-U-C^{14}	Additions: 20 mmolar DL-glutamate-1-C^{14}	Additions: 20 mmolar DL-glutamate-1-C^{14} +10 mmolar glucose
3EL (chloride) added	Control	Q_{O_2}	11.1	12.6	13.2
		Uptake	259	225	416
		$C^{14}O_2$	182	411	346
	10^{-4} *M*	Q_{O_2}	2.3	3.9	2.6
		Uptake	180	208	197
		$C^{14}O_2$	38	166	105
	10^{-5} *M*	Q_{O_2}	4.0	5.6	4.6
		Uptake	184	195	195
		$C^{14}O_2$	43	309	146
	10^{-6} *M*	Q_{O_2}	6.2	7.8	5.7
		Uptake	192	224	214
		$C^{14}O_2$	62	385	252
	10^{-7} *M*	Q_{O_2}	12.4	13.2	13.8
		Uptake	241	247	425
		$C^{14}O_2$	173	383	312
3ET (sulphate) added	10^{-4} *M*	Q_{O_2}	2.1	3.6	2.6
		Uptake	198	211	202
		$C^{14}O_2$	24	132	87
	10^{-5} *M*	Q_{O_2}	9.2	10.5	11.1
		Uptake	213	232	312
		$C^{14}O_2$	136	367	289

NOTE: Incubation: 60 minutes; gas phase, oxygen.
Uptake: Figures are total activities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. introduced per vessel.
$C^{14}O_2$: Figures are total activities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. introduced per vessel.

TABLE X

The effect of mixtures of the triethyl and tetraethyl derivatives of lead and tin on some metabolic activities of rat brain cortex slices

Additions		Additions: 10 mmolar glucose-U-C^{14}	Additions: 20 mmolar DL-glutamate-1-C^{14}	Additions: 20 mmolar DL-glutamate-1-C^{14} +10 mmolar glucose
Control	Q_{O_2}	12.0	12.3	13.1
	Uptake	205	231	428
	$C^{14}O_2$	171	396	295
10^{-4} *M* 4EL + 10^{-6} *M* 3EL	Q_{O_2}	5.8	6.9	6.2
	Uptake	181	216	232
	$C^{14}O_2$	74	252	149
10^{-4} *M* 4ET + 10^{-6} *M* 3ET	Q_{O_2}	8.2	7.9	6.8
	Uptake	192	201	216
	$C^{14}O_2$	101	295	186

NOTE: Incubation: 60 minutes; gas phase, oxygen.
Uptake: Figures given are total radioactivities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. substrate introduced per vessel.
$C^{14}O_2$: Figures are total activities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. substrate introduced per vessel.

Experiments, the results of which are given in Tables XI and XII, demonstrate the following facts:

(*a*) The respiration of rat brain cortex slices, which have been exposed to phospholipase A for 30 minutes, becomes exceedingly sensitive to 10^{-4} *M* 4EL. Under these conditions almost complete abolition of the respiration takes place. The respiration of rat brain cortex slices exposed to 10^{-4} *M* 4EL for 30 minutes has much less sensitivity to phospholipase A (Table XI).

TABLE XI

The effects of 4EL and heated venom on glucose oxidation by rat brain cortex slices

Additions at zero time	Additions after 30 minutes' incubation					
	Nil		Heated venom (100 γ in 3 ml)		10^{-4} *M* 4EL	
	Q_{O_2}	$C^{14}O_2$	Q_{O_2}	$C^{14}O_2$	Q_{O_2}	$C^{14}O_2$
Nil	9.9	153	8.4	160	9.2	146
Heated venom (100 γ in 3 ml)	5.2	96	—	—	1.3	21
10^{-4} *M* 4EL	9.6	142	4.9	62	—	—

NOTE: Incubation: 90 minutes; gas phase, oxygen; all vessels contained 10 mmolar glucose at zero time; a trace amount of glucose-U-C^{14} was added to the main compartment of the vessel at 30 minutes, incubation.
Q_{O_2}: The oxygen uptake values are for the 30- to 90-minute interval of incubation.
$C^{14}O_2$: Values are total activities of $BaC^{14}O_3$ as c.p.m. per mg dry weight of tissue per 10^5 c.p.m. introduced for the 30- to 90-minute interval of incubation.
All figures are averages of results of three experiments.

(*b*) The presence of phospholipase A greatly diminishes the active uptake of an amino acid (alanine), its effect being but little enhanced by the addition of 10^{-4} *M* 4EL (Table XII). Moreover, the depressing effect of phospholipase A on the formation of $C^{14}O_2$ from alanine-1-C^{14} is much increased in presence of glucose.

These results are to be expected if phospholipase A can destroy, or interfere with, phospholipids necessary for active transport of amino acids and of cations across the neuronal membrane, and if 4EL (and 4ET) has a similar (but less vigorous) effect under the conditions of these experiments. The high sensitivity to 4EL of potassium-stimulated brain respiration would then be due to a block of cation transport, thus abolishing cationic stimulation. On the other hand, the diminution of the respiration of phospholipase-treated brain slices may indicate an accelerated loss of substances necessary for optimal neuronal respiration from the slices, a phenomenon known to occur in brain tissue as a result of phospholipase treatment (14). It is unlikely that the increased sensitivities are due to increased permeability to 4EL, as the latter at a concentration of 10^{-4} *M* was found to have no inhibitory effect on the respiration of rat brain mitochondria or rat brain homogenates.

The Metabolism of Brain Cortex Slices from Rats Poisoned with Tetraethyl Lead and Tetraethyl Tin

Experiments have been carried out to compare the in vitro results, obtained

TABLE XII

The effect of 4EL and heated venom on some metabolic activities of rat brain cortex slices

Additions	10 mmolar glucose-U-C^{14}		4 mmolar DL-alanine-1-C^{14}			4 mmolar DL-alanine-1-C^{14} +10 mmolar glucose		
	Q_{O_2}	$C^{14}O_2$	Q_{O_2}	Uptake	$C^{14}O_2$	Q_{O_2}	Uptake	$C^{14}O_2$
Control	12.0	169	7.2	357	243	11.9	840	438
150 γ heated venom in 3 ml	8.9	127	4.4	184	117	7.9	277	58
10^{-4} *M* 4EL	13.1	181	6.3	306	231	12.4	386	419
150 γ heated venom in 3 ml +10^{-4} *M* 4EL	4.5	68	3.9	160	118	4.9	240	40

NOTE: Incubation: 60 minutes; gas phase, oxygen.
Uptake: Figures are total activities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^3 c.p.m. introduced.
$C^{14}O_2$: Figures are total activities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^3 c.p.m. introduced.

TABLE XIII

The metabolism of brain cortex slices from rats treated with tetraethyl tin (4ET)

Number of injections	Substance injected		Additions			
			10 mmolar glucose-U-C^{14}	20 mmolar DL-glutamate-1-C^{14}	20 mmolar DL-glutamate-1-C^{14} +10 mmolar glucose	2 mmolar glycine-1-C^{14} +10 mmolar glucose
2	Control	Q_{O_2}	11.7	12.5	13.2	12.3
		Uptake	206	226	441	1560
		$C^{14}O_2$	174	396	271	145
	4ET	Q_{O_2}	11.9	10.2	10.9	12.6
		Uptake	220	226	325	1790
		$C^{14}O_2$	167	378	226	—
4	Control	Q_{O_2}	10.7	11.9	12.7	12.5
		Uptake	232	290	422	1820
		$C^{14}O_2$	182	402	290	—
	4ET	Q_{O_2}	11.6	6.1	5.7	9.0
		Uptake	205	214	266	860
		$C^{14}O_2$	190	207	166	—

NOTE: Injections were given intraperitoneally, one each day previous to the experiment and the last one 2 hours before the experiment. 4ET was given at 10 mg kilo body weight, dissolved in propanol, and the solution diluted with water so that the final concentration of propanol was 3% v/v. All injections were 2 ml.

Incubation: 60 minutes; gas phase, oxygen.

Uptake: Figures are total activities of 80% ethanol extracts in c.p.m. per mg dry weight of slice per 10^5 c.p.m. introduced.

$C^{14}O_2$: Figures are total activities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. introduced.

Figures are averages of results of at least four experiments.

TABLE XIV

Metabolism of brain cortex slices from rats treated with tetraethyl lead (4EL)

Number of injections	Substance injected		Additions			
			10 mmolar glucose-U-C^{14}	20 mmolar DL-glutamate-1-C^{14}	20 mmolar DL-glutamate-1-C^{14} +10 mmolar glucose	2 mmolar glycine-1-C^{14} +10 mmolar glucose
4	Control	Q_{O_2}	10.9	11.8	12.3	11.7
		Uptake	189	249	436	1530
		$C^{14}O_2$	182	398	252	—
	4EL	Q_{O_2}	11.9	11.0	11.3	12.6
		Uptake	209	204	291	1120
		$C^{14}O_2$	184	315	195	—
6	Control	Q_{O_2}	11.2	12.3	13.1	12.1
		Uptake	231	216	409	1680
		$C^{14}O_2$	192	381	268	—
	4EL	Q_{O_2}	12.2	10.1	9.8	10.9
		Uptake	226	208	276	740
		$C^{14}O_2$	189	298	156	—

NOTE: Injections were given intraperitoneally, one each day previous to the experiment and the last one 2 hours before the experiment. 4EL was given at 10 mg kilo body weight, dissolved in propanol, and the solution diluted with water so that the final concentration of propanol was 3% v/v. All injections were 2 ml.

Incubation: 60 minutes; gas phase, oxygen.

Uptake: Figures are total activities of 80% ethanol extracts in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. substrate introduced per vessel.

$C^{14}O_2$: Figures are total activities of $BaC^{14}O_3$ in c.p.m. per mg dry weight of tissue per 10^5 c.p.m. of substrate introduced per vessel.

Figures are averages of at least two experimental results.

with 4EL and 4ET, with those found in the intact animal after administration of these substances.

Rats were poisoned with daily intraperitoneal injections of 10 mg per kilo body weight of either 4EL or 4ET in a 3% (v/v) propanol solution. Control animals received the same number of 3% propanol injections. After the first three to four injections, typical symptoms started to appear with the poisoned animals, such as a decrease in body weight (probably due to a lower food intake), difficulty in breathing, and a partial paralysis of the posterior extremities. After a specified number of injections the animals were killed and their brains excised, sliced, and incubated in the usual manner. The results obtained with 4ET are given in Table XIII. It can be seen from these results that after two injections of 4ET, when symptoms of intoxication were not yet apparent, the in vitro metabolic activities of brain slices were not appreciably different from those of the controls. The only significant difference was a partial inability of slices from poisoned animals to concentrate glutamic acid. After four injections, however, when symptoms were well established, the results presented a picture strikingly similar to the one obtained when 4ET was added in vitro. Glucose metabolism seemed to be undisturbed at least as far as the metabolic activities measured were concerned. There was a considerable decrease in the rate of oxygen uptake with glutamate or with a mixture of glucose and glutamate as substrates comparable with the inhibitions obtained with 4ET added in vitro and reported above. Another clear effect was a failure of the slices from poisoned animals to accumulate either glutamic acid or glycine against the diffusion gradient. Thus, under our conditions, and for the metabolic processes studied, the metabolism of brain slices from rats poisoned with 4ET was similar to, if not identical with, that obtained with normal brain in the presence of a 10^{-4} *M* 4ET.

Results obtained with brains from animals poisoned by 4EL, reported in Table XIV, show that the same close correlation of in vivo and in vitro findings observed with 4ET can also be obtained with 4EL. With 4EL, however, it was necessary to poison the animals for a longer period of time in order to obtain definite effects. After six injections a lessened ability of the slices to accumulate glutamic acid or glycine became apparent. The decrease in rate of oxygen uptake when glutamate, or a mixture of glutamate and glucose, was present as substrate was not as clear as with 4ET. An important observation is that with both poisons the effects observed on brain slices appear at approximately the same time as the typical symptoms of intoxication.

Discussion

The salient properties of 4EL (and 4ET), so far as their effects on brain metabolism in vitro are concerned, are as follows:

(1) Inhibition of active (energy-dependent) transport of amino acids,

(2) Inhibition of potassium-stimulated respiration in presence of glucose, with little or no effect on the unstimulated respiration.

(3) Enhancement by 4EL of the rate of decrease of respiration with rat brain cortex slices treated with phospholipase A. The energy-dependent transport of amino acid into the tissue is almost completely abolished by the enzyme, so that little enhancing effect of 4EL is possible.

The results as a whole may be satisfactorily explained if there is a combination or association of 4EL and 4ET with phospholipid groups of transport carriers located at the brain cell membranes. They could, in this manner, abolish the abilities of the carriers to transport cations or amino acids into the cell interior and thus bring about the phenomena described in this paper.

It is of importance to note that when brain is poisoned in the living animal by administration of tetraethyl lead the only metabolic abnormality which has been found is an inability of brain slices to transport actively glutamic acid and glycine. Since this disturbance coincides with the appearance of pathological symptoms it is reasonable to assume that some of the neurological symptoms of tetraethyl lead intoxication may be a consequence of the failure of the brain cell to transport amino acids (and possibly other ions) across the neuronal membrane. It is, however, possible that the modification of the cell membrane due to a combination with 4EL may exert other effects, e.g. increased leakage of components from the cells, thus bringing about a variety of neurological disturbances.

The effects of tetraethyl tin on the metabolism of the central nervous system both in vivo and in vitro seem to be very similar to those of tetraethyl lead.

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COMPARISON BETWEEN SEASONAL AND THERMAL ACCLIMATION IN WHITE RATS[1]

V. METABOLIC AND CARDIOVASCULAR RESPONSE TO NORADRENALINE

O. HÉROUX

With the technical assistance of DONNA WRIGHT

Abstract

White rats cold-acclimated at a constant temperature in the laboratory are known to be more sensitive to noradrenaline than warm-acclimated controls. The present study reveals that this responsiveness to noradrenaline is linearly related to the temperature at which the animals are conditioned. Concomitant measurements (after intramuscular injection of noradrenaline) of oxygen consumption, blood pressure, and heart rates on white rats acclimatized to outdoor summer or winter conditions revealed a much greater metabolic and cardiovascular sensitivity to noradrenaline in winter than in summer rats. The slight degree of shivering upon exposure to 6° C which was observed in the outdoor winter rats, as well as their great sensitivity to noradrenaline, suggests that under both indoor and outdoor environmental conditions, increased cold resistance is obtained through similar metabolic mechanisms.

Both indoor and outdoor cold-exposed rats develop an increased capacity to produce heat and an increased non-shivering thermogenesis (1). Under the laboratory conditions of constant low temperature the metabolic adjustments were accompanied by a reduced adrenal cortex activity and an increased thyroid activity (2, 3), while under the fluctuating environmental conditions prevailing outdoors they were accompanied by an increased adrenal cortex activity and a reduced thyroid activity (2, 3).

To separate the adjustments which are essential for metabolic acclimation to cold from those which are specific to the particular environmental conditions under which the animals are conditioned, the metabolic and cardiovascular responses to noradrenaline which have been well demonstrated in indoor cold-acclimated rats (4, 5, 6) were measured in outdoor cold-acclimated rats. For control purposes they were also measured on rats exposed indoors to different constant temperatures (30° C, 25° C, 19° C, and 6° C).

Experimental Procedures

Animals and Environmental Conditions

Adult male Sprague–Dawley rats were submitted to two different sets of environmental conditions for an average period of 105 days (92–118 days): (*a*) in rooms maintained at a constant temperature (30° C, 25° C, 19° C, and 6° C); and (*b*) outdoors exposed in groups of 10 per cage to the fluctuating en-

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vironmental conditions from May 30 to August 29, 1960, during the summer and from October 21, 1959, to January 20, 1960, during the winter. Ten rats among the winter group were left outdoors until May 3, when the mean temperature was about +5° C. At that time the sensitivity of these rats to noradrenaline was tested following the procedure that will be described below. In August 1960 the average day temperature was 19° C; in January 1960 it was −10° C.

The feeding and housing conditions have been described in a previous paper (1). The average weight of the rats at the beginning of the acclimation period was 228 g for the outdoor rats and 200 g for the indoor ones.

Methods

After acclimation, shivering at 6° C was measured on a certain number of summer and winter acclimatized rats according to the method described previously (7).

Following this test, the animals were anesthetized with sodium barbital (290 mg/kilo i.p.). One and a half hours later, at a room temperature of 24° C, blood pressure was measured by an occlusion plethysmograph on the tail and O_2 consumption, with a head mask associated with a Pauling O_2 analyzer and a dry test meter which were the components of an open-circuit system. The PO_2 and volume of CO_2-free outlet gas were recorded and the appropriate equation corresponding to these conditions (8) was used to calculate the O_2 consumed per unit time.

After O_2 consumption and blood pressure were measured for 10 minutes, a 0.2 mg/kg dose of noradrenaline bitartrate 0.2% (Levophed, Winthrop Laboratories of Canada Limited) was injected in the gastrocnemius muscle.

In all experiments oxygen consumption and blood pressure were then measured for 60 minutes after the injection, readings being taken every minute during the first 10 minutes and every 5 minutes thereafter.

Due to variations in the response pattern observed in different animals, the effect of noradrenaline on the electrical activity of muscle (EMG) and on the oxygen consumption was estimated in the following way. The EMG values of each rat (10 μv = 1 cm) and the O_2 consumption (1 ml O_2 = 1 cm) were plotted against time (5 minutes = 1 cm) on metric paper and the areas under the curves were measured with a planimeter. In these units an EMG response to cold exposure of 1 cm^2 would correspond to an average increase of 1.25 μv/minute during a period of 40 minutes; a calorigenic response to noradrenaline of 1 cm^2 would correspond to an average increase of 0.0833 ml/minute of O_2 for a period of 60 minutes. Colonic temperatures were measured throughout with a copper–constantan thermocouple inserted to a depth of 5 cm in the rectum.

On the rats tested in May, a rise in body temperature was partly prevented by covering the animal with a copper jacket through which cold water was circulated. In these animals, muscle electrical activity as well as heart rates was continuously recorded for the first 10 minutes and every 5 minutes there-

after. The electrical potentials were picked up by electrodes attached to the skin according to the method previously described (7).

The depth of anesthesia after barbitone injection was estimated by the righting reflex in 12 rats acclimated at 30° C and 12 rats acclimated at 6° C. The test consisted simply of measuring the time taken for the righting reflex to disappear after injection of the anesthetic and the time required for it to reappear. The righting reflex is present when the animal can turn over after being laid on its back.

Results

Shivering

As previously demonstrated in rats exposed outdoors under similar conditions, winter rats shivered much less than summer rats upon exposure to 6° C.

The shivering response, increase in muscle electrical activity, during the test period of 40 minutes was on the average 11.4 ± 2.1 μv in winter rats and 43.0 ± 4.2 μv in summer rats.

Metabolic Response to Noradrenaline

After injections of 0.2 mg/kg of noradrenaline bitartrate, both winter (January) and spring (May) rats had a much greater metabolic response than the summer rats (Fig. 1). Although the average outdoor temperature during the day was much higher in May than in January, no significant differences were found in the responses to noradrenaline between spring and winter rats.

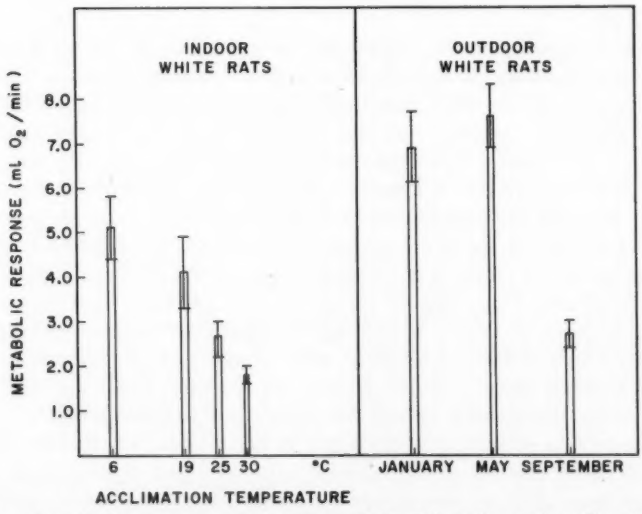


FIG. 1. Metabolic responses to intramuscularly injected noradrenaline bitartrate (0.2 mg/kg) in white rats acclimated indoors to different temperatures and outdoors to different seasons. Cross bars at the top of the column indicate the standard errors of the mean responses.

However, during the night in April, the temperature went down regularly to about 0° C. The indoor cold-acclimated rats showed a similar increase in their sensitivity to noradrenaline. This response, as shown by the results obtained on different groups of rats acclimated to different constant temperatures, was linearly related to the acclimation temperature (Fig. 1); the lower the temperature the greater was the response. The equation characterizing this relationship is $Y = 74.5 - 10.5\ X$, Y being the metabolic response and X the acclimation temperature.

The calorigenic response to noradrenaline occasioned an increase in colonic temperature of only 0.05° C on the average in the outdoor summer rats but in the winter rats the colonic temperature rose by 1.5° C. In the rats tested in May, the use of the cool jacket prevented the colonic temperature from rising by more than 0.9° C. In the indoor rats, the colonic temperature went up by 0.5° C in the 30° C-acclimated rats and 1.7° C in the 6° C rats.

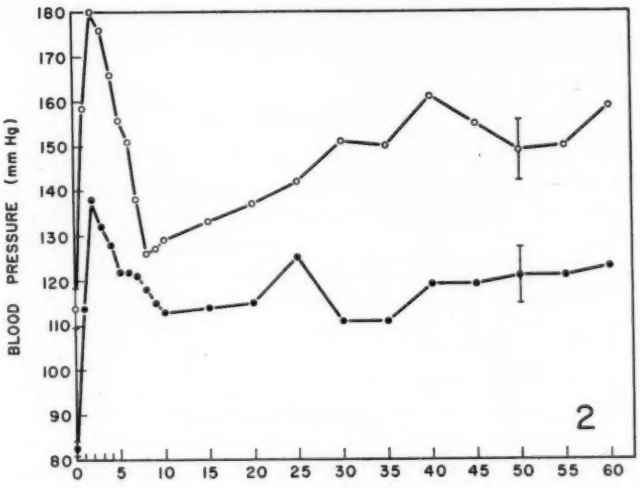
In the rats tested in May, in which the calorigenic response to noradrenaline and EMG were simultaneously measured at room temperature, muscle activity was at its minimum throughout the experiment. Noradrenaline, which almost tripled the oxygen consumption, had no effect on muscle activity.

Pressor Response to Noradrenaline.

Before injection of noradrenaline there was a statistically significant difference in the initial blood pressure between warm- and cold-conditioned rats. In both indoor and outdoor rats the "cold" animals had a higher blood pressure than their warm controls, the greatest difference being found in the indoor rats (Fig. 2).

Within 2 minutes of the intramuscular injection of noradrenaline, blood pressure increased on the average to a level of 138 mm Hg in the "30° C rats" and 180 mm Hg in the "6° C rats" (Fig. 2); in the outdoor rats it increased to 148 mm Hg in the summer rats and 198 mm Hg in the winter rats (Fig. 3). The peripheral vasoconstriction that brought about the increase in blood pressure was immediately followed in all groups by a reflex vasodilation which gradually lowered the blood pressure in the following 8 minutes to an average level of 115–125 mm Hg in all groups. In the next 50 minutes blood pressure gradually increased to reach a plateau at a level of 120 mm Hg in the "30° C rats" and 155 mm Hg in the "6° C rats". In the outdoor rats, blood pressure reached a plateau at a level of 125 mm Hg in the summer and 155 mm Hg in the winter. This difference between warm- and cold-conditioned rats in the plateau at which blood pressure leveled off reflected the initial difference in blood pressure that existed before the injection of noradrenaline.

The recordings of heart rate obtained on one group (the outdoor rats tested in May) showed that the initial increase in blood pressure was closely followed by a significant drop in heart rate (Fig. 4). An extrapolation of the blood pressure curve reveals that the maximum increase was attained, on the average, around 1.5 minutes after injection, whereas the lowest level for heart rate was



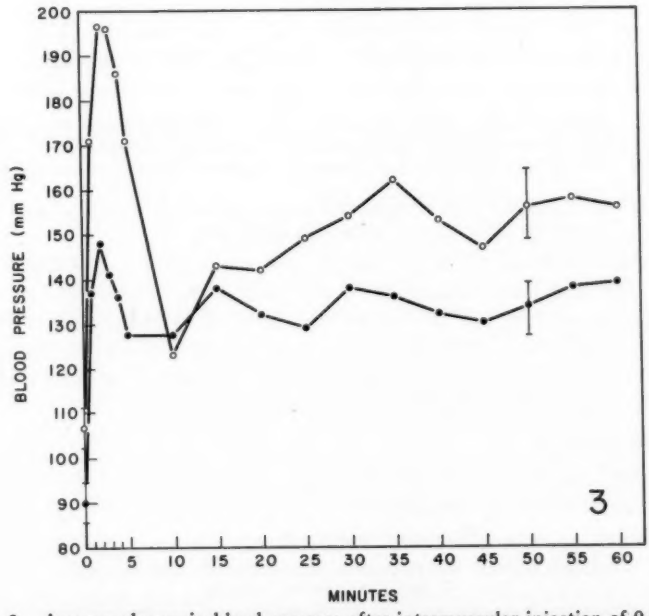


FIG. 2. Average change in blood pressure after intramuscular injection of 0.2 mg/kg of noradrenaline bitartrate in 30° C acclimated (●) and in 6° C acclimated rats (○). There were 12 rats in each group. The cross bars indicate the standard errors.

FIG. 3. Average change in blood pressure after intramuscular injection of 0.2 mg/kg of noradrenaline bitartrate in white rats acclimatized to outdoor summer conditions (●) and to winter conditions (○). There were 9 rats in the first group and 14 in the second. Cross bars indicate standard errors.

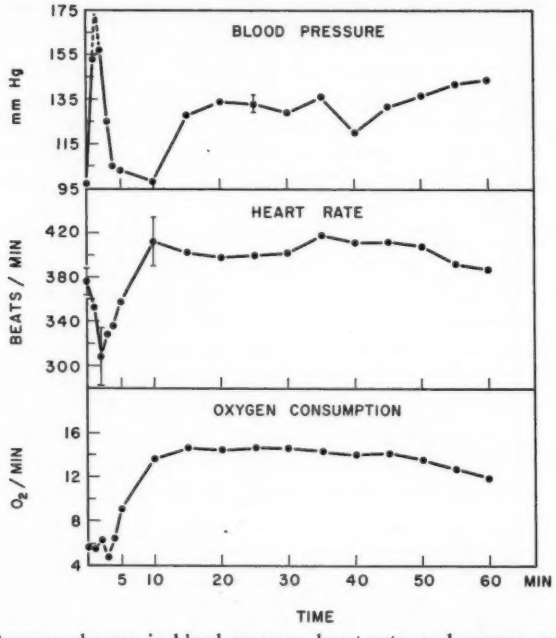


FIG. 4. Average changes in blood pressure, heart rate, and oxygen consumption in outdoor rats tested in May after intramuscular injection of 0.2 mg/kg of noradrenaline bitartrate. There were five rats in this group. Cross bars indicate the standard errors.

reached after 2 minutes. As blood pressure started dropping, heart rate increased, returning gradually to the initial level (Fig. 4).

After the 10th minute, blood pressure increased again to a level of 135 mm but this time it did not bring about a lowering of the heart rate. At the 10th minute the heart rate had reached a high level of 412 beats/minute where it remained more or less constant for the following 40 minutes. It started dropping only in the last 10 minutes of the experiment. Both heart rate and oxygen consumption generally followed the same trend (Fig. 4).

Depth of Anesthesia

In both 30° C and 6° C acclimated rats, it took about the same time after injection of barbitone for the righting reflex to disappear as well as to reappear

TABLE I

Disappearance and reappearance times for the righting reflex in barbital-anesthetized rats

Groups	n	Disappearance time (minutes)	Reappearance time (minutes)
30° C	(12)	20.0±3.7*	579±37.6
6° C	(12)	23.6±4.0	553±12.1

NOTE: Number of animals in parentheses.
*Mean ± S.E.M.

later on, which indicates that both groups reached the same depth of anesthesia in about the same time and remained anesthetized the same length of time (Table I).

Discussion

The observation of a similar degree of anesthesia in 30° C and 6° C acclimated rats eliminated the possibility that the difference in the metabolic and cardiovascular responses between warm- and cold-acclimated rats was due to a difference in depth of anesthesia.

The observation that just as large an increase in O_2 consumption could be obtained with noradrenaline in animals in which body temperature was prevented from rising by more than 0.9° C as in winter rats or 6° C acclimated rats in which body temperature rose by 1.5° C and 1.7° C, respectively, eliminates the possibility that the large increase in O_2 consumption found in the cold-conditioned rat was mostly a Q_{10} effect due to the severe increase in body temperature.

The present results confirm and extend the studies of Hsieh and Carlson (4) and Depocas (5). The metabolic response to noradrenaline, at least in the indoor rats, was found to be linearly related to the temperature at which the animals had been acclimated. This relationship does not seem to hold for outdoor rats, however, since rats tested in May, when the average temperature during the day (for 24 hours) was +5° C, reacted just as much as the rats tested in January when the average day temperature was −10° C. It could be, however, that a greater degree of huddling took place in January than in May with the result that the degree of cold exposure by each individual rat was about the same in both months. In any event, both in January and May, outdoor rats had a much greater metabolic response to noradrenaline than 6° C acclimated rats, which suggests that the degree of cold experienced by these rats was greater than the one experienced by rats kept at 6° C unless there are other factors involved in this response besides temperature.

The blood pressure results also confirm and extend similar observations reported by LeBlanc (6). They show that noradrenaline produced a much greater vasoconstriction of the arterioles in the cold-conditioned rats than in the warm controls, either indoor or outdoor. The sudden increase in blood pressure quickly stimulated the parasympathetic system with the result that the peripheral vessels vasodilated, the heart rate dropped, and the blood pressure decreased. The blood pressure drop was much greater in the two cold-conditioned groups than in the warm controls, which was to be expected since the stimulus for the vasodilation reflex, that is the initial degree of vasoconstriction, was much greater in the "cold" animals. The greater sensitivity of the cardiovascular system of the "cold" rats is also demonstrated by the higher level at which blood pressure leveled off during the last 30 minutes of the experiments.

The large increase in oxygen consumption found in the "cold" rats was accompanied, as expected, by a significant increase in the heart rate and in

the blood pressure. The fact that blood pressure, heart rate, and oxygen consumption were maintained more or less constant for at least 40 minutes probably reflects a continuous and regular muscular absorption of the injected noradrenaline once the peripheral circulation had reached a new equilibrium after the severe shock of the first few minutes.

The slight degree of shivering upon exposure to 6° C which was observed in the outdoor winter rats as well as their great metabolic and cardiovascular sensitivity to noradrenaline, reveals that, under the natural fluctuating environmental conditions prevailing outdoors during the winter, white rats kept in groups of 10 per cage develop the same type of metabolic adjustments as those found in indoor cold-acclimated rats. Under these outdoor conditions as well as under the indoor laboratory conditions, the cold resistance of white rats is increased through the development of non-shivering thermogenesis mediated by noradrenaline.

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TRACE ELEMENTS IN TRANSPLANTED TUMORS[1]

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Abstract

Spectrographic analyses were made of three transplanted tumors and two normal tissues of mice and hamsters. Significant differences, both qualitative and quantitative, were found among the tumors and between tumor and normal tissue.

Boron and molybdenum were characteristic of the melanoma and were undetected or most severely reduced in normal muscle, leukemia, and sarcoma. Chromium was a significant component of the melanoma-host liver, but was not detected in other livers. Nickel was found in the normal liver, but was lacking from the melanoma-host and leukemia-host livers.

For many essential enzymes, trace elements serve as molecular components or as cofactors (1, 2). Trace element distribution in tissue should reflect the enzyme patterns of the tissue and provide a convenient tool for the study of enzyme changes in carcinogenesis. This tool has not been exploited extensively, and there is a dearth of accurate analyses of genetically and nutritionally uniform populations.

To provide a base line for such investigations of neoplasia, the trace element composition of two mouse tumors, one hamster tumor, and selected tissues of normal and tumor-bearing animals were studied spectrographically.

Materials and Methods

Three tumors were selected to represent the major classes, carcinoma, leukemia, and sarcoma. They also provided a graded variation in the proportion of tumor cells to stroma; with the most extensive stroma in the mouse melanoma, B-16, and the least in the mouse lymphatic leukemia, P-1534. Both the melanoma and the leukemia were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and were maintained in tumor strain animals obtained from the same laboratory. The hamster sarcoma used was the transplantable, methyl-cholantrene-induced tumor obtained from the Department of Biology of Boston University through the courtesy of Dr. D. I. Patt (3).

The melanoma was transplanted to 100 C57/B1 mice, with 85% success, and liver and tumor tissues were harvested after 4 weeks. The leukemia was

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transplanted to 100 AKD_2F_1/Jax mice, from which tumor and liver tissues were removed after 3 weeks. The Boston University sarcoma was grown in the flanks of 10 hamsters and harvested after 6 weeeks of growth.

TABLE I

Details of the spectrographic method

Spectrograph	JACO 21-foot grating, Wadsworth mount; first-order dispersion 5.2 Å/mm
Condensing optics	25 cm focal length cylindrical lens (horizontal axis) at the slit; 6.7 cm focal length cylindrical lens (vertical axis) 16.1 cm from the slit; diaphragm with 5 mm aperture 27.5 cm from the slit; 10 cm focal length spherical lens 58.1 cm from the slit; arc located 72.5 cm from the slit
Slit width	30 microns
Intensity control	7-step sector at the slit, long intensity ratio = 0.2; 1-mesh screen for further reduction
Slit length	6 mm; sector height adjusted for steps 3, 4, 5
Electrodes	National Carbon Co. graphite (Special Grade): sample (anode), ⅛ in. rod, plain crater ⅛×⅜ in.; counter-electrode, ⅛ in. rod
Emulsions and range	Eastman Kodak Type S.A.1 plate, 2200–3550 Å; Eastman Kodak Type III-F plate, 3550–4900 Å
Processing	D-19 developer, 3 minutes, 20° C; stop bath 15 seconds; acid fix 20 minutes; wash 30 minutes
Voltage	220 volts d-c. on open circuit
Exposure	Completion; 80 to 90 seconds
Current	Started at 4, raised to 7 amperes
Air jet	1 cubic liter/minute
Sample	1 part of sample to 1 part of SA matrix to 2 parts of graphite powder containing 0.1% $PdCl_2$
Matrix SA	Prepared from Johnson-Matthey "Specpure" compounds; 33% SiO_2 and 67% Al_2O_3
Artificial standards	Prepared from Johnson-Matthey "Specpure" compounds; matrix of KCl, 10 parts; NaCl, 10 parts; Fe_2O_3, 5 parts; MgO, 5 parts; $CaCO_3$, 5 parts; Al_2O_3, 3 parts; SiO_2, 2 parts: to this matrix was added "Specmix" (Spex Industries) containing 43 metals at 1.34% concentrations and standards were prepared by dilution to cover the range 3.16 to 10,000 p.p.m.
Photometry	ARL photodensitometer used; galvanometer readings between 1 and 95 were used and background corrections applied in all cases; clear-plate readings adjusted to 100 before each measurement; readings made on step 4
Exposure sequence	At least one standard exposed on each sample plate to check calibration curves and minimize working-curve drift; samples analyzed in triplicate, one on each of three pairs of plates

Tissues were frozen in acetone–dry ice and stored at −4° C. After being thawed and washed with distilled water, tissues of two or three individuals were pooled and ashed in platinum crucibles at 600° C in an electric muffle furnace. The ash was stored in plastic vials, as 85 samples: 32 of melanoma, 20 melanoma-bearing liver, 14 leukemia, 9 leukemia-bearing liver, 6 hamster sarcoma, 2 normal mouse muscle, and 2 normal mouse liver.

The ash samples were analyzed on the 21-ft Jarrell-Ash grating spectrograph, under the conditions shown in Table I and with the precision shown in Table II.

TABLE II

Relative errors of the method

Spectral line	Internal standard line	Relative error (E), %
B 2497	Pd 2476	20.3
Mn 2576	Pd 2476	12.1
Mo 3193	Pd 3258	12.0
Cu 3273	Pd 3258	8.8
Zn 3282	Pd 3258	2.8
Ni 3414	Pd 3258	21.5
Cr 4254	Pd 3258	10.4

NOTE: E calculated by the method of Shaw and Bankier (4),

$$E = \frac{100}{2} \times \frac{\text{antilog } (\bar{x} + s_{x0}) - \text{antilog } (\bar{x} - s_{x0})}{\text{antilog } \bar{x}} \%,$$

where s_{x0} is the standard deviation and $\bar{x}$ the mean of log k.

Results

The triplicate analyses of each sample were highly reproducible. Several elements were found in high concentration, above 1000 p.p.m., in all tissues including iron (Fe), calcium (Ca), potassium (K), phosphorus (P), magnesium (Mg), and sodium (Na). Platinum (Pt), detected in all samples, was considered contamination from the crucibles.

Among the three types of tumor analyzed, the main qualitative differences lay in the elements boron (B) and molybdenum (Mo). Both elements were detected in the B-16 melanoma, with an average concentration of 21 p.p.m. for B and 82 p.p.m. for Mo. B was detected as a trace, less than 5 p.p.m., in the P-1534 leukemia, in which no Mo was detected. Normal mouse muscle did not show any detectable amount of B or Mo. The concentration of Mo in the B-16 tumor varied over a wide range, from 40 to 190 p.p.m., while B showed a comparatively uniform distribution, of 5 to 30 p.p.m.

Among the three types of liver, the distinctive qualitative difference was in chromium (Cr), detected only in the livers of melanoma-bearing animals, with a range of 5 to 210 p.p.m. Nickel (Ni) was found in normal liver, with an average concentration of 15 p.p.m., but was not detected in livers of melanoma-bearing or leukemic mice. Ni was detected in trace amounts, less than 5 p.p.m., in all three tumors, and was present at 180 p.p.m. in normal mouse muscle.

Average values of the triplicate analyses of individual samples are tabulated in Tables III through VIII. The average values for each tissue are shown in Fig. 1. The significance of differences in element concentrations among the tissues was tested statistically (4) (with σ^2 unknown and $\alpha = 0.01$), with results shown below under each element:

Boron.—B was detected only in the B-16 melanoma and the P-1534 leukemia, with concentration differences between the two tumors shown significant by the "t" test.

Manganese.—Differences in Mn concentration among liver, muscle, and

TABLE III

Melanoma B-16
(Average values, in p.p.m., for individual sample)

Sample	Element						
	B	Mn	Mo	Cu	Zn	Ni	Cr
MT 83, 84	18	14	135	93	tr.	13	330
MT 50, 51	30	13	57	78	380	tr.	tr.
MT 63, 64	19	10	47	81	420	tr.	n.d.
MT 65, 66	15	14	45	76	380	tr.	tr.
MT 59, 60	18	16	41	76	290	tr.	43
MT 73, 74	22	10	73	95	330	tr.	44
MT 67, 68	tr.	12	125	60	430	tr.	40
MT 71, 72	21	22	88	85	310	5	125
MT 52, 53	21	29	71	>200	483	tr.	140
MT 77, 78	20	13	70	55	337	tr.	69
MT 81, 82	17	17	91	67	460	19	245
MT 56, 57	25	16	77	72	435	tr.	tr.
MT 79, 80	22	13	75	71	340	7	210
MT 61, 62	28	11	49	73	387	tr.	tr.
MT 89, 90	24	13	97	57	340	tr.	104
MT 75, 76	28	16	93	44	523	tr.	220
MT 106	tr.	12	42	59	320	tr.	tr.
MT 87, 88	14	15	121	59	460	15	192
MT 93, 94	18	12	90	91	420	n.d.	tr.
MT 103	tr.	13	39	64	317	n.d.	tr.
MT 58	21	21	111	102	487	14	233
MT 99, 100	tr.	17	83	34	397	tr.	51
MT 69, 70	20	17	68	80	tr.	n.d.	tr.
MT 91, 92	24	14	59	65	470	n.d.	n.d.
MT 101, 102	15	13	79	76	453	tr.	79
MT 117	16	10	93	78	425	n.d.	tr.
MT 108	tr.	22	41	65	tr.	tr.	52
MT 55	29	16	159	21	487	n.d.	tr.
MT 85, 86	18	27	77	58	330	tr.	477
MT 54	21	13	69	54	403	n.d.	tr.
MT 111	25	15	71	74	350	tr.	50
MT 115	tr.	13	191	90	410	tr.	41

NOTE: n.d. = not detected; tr. = trace, less than 5 p.p.m.

tumor were significant, but differences among the three types of tumor were not. The hamster sarcoma had significantly higher concentrations of Mn than either of the other tumors, which were not significantly different.

Molybdenum.—The three types of liver had similar concentrations of Mo at the 0.01% level, with significant differences at the 0.1% level. The concentrations in the B-16 melanoma varied widely but were significantly higher than in liver. This element was not found in normal muscle or in the other two tumors.

Copper.—Similar high concentrations, greater than 200 p.p.m., were detected in normal liver, leukemic liver, and the hamster sarcoma. Melanoma-bearing liver differed significantly from normal muscle, melanoma, and leukemia, which had similar mean values. The leukemic population showed wide variation in copper concentration.

Zinc.—This element was detected in considerable amounts in all tissues. Liver from melanoma-bearing mice showed the highest concentration, 1400

p.p.m., significantly different from all other tissues. All other tissues were significantly different from each other, with the exception of normal and leukemic liver, which were similar. Among the tumors, the hamster sarcoma had the highest concentration, 950 p.p.m.

Nickel.—Qualitative differences were found between normal liver and liver from cancerous hosts. Normal liver differed significantly from normal muscle,

TABLE IV

Liver from melanoma-bearing hosts
(Average values, in p.p.m., for individual sample)

Sample	Element				
	Mn	Mo	Cu	Zn	Cr
MT 77, 78	87	24	126	1400	171
MT 71, 72	123	23	76	1500	tr.
MT 67, 68	52	22	>200	1713	36
MT 69, 70	51	21	80	1260	tr.
MT 103	68	19	>200	1067	64
MT 52, 53	81	25	79	1460	tr.
MT 79, 80	59	17	85	1113	191
MT 56, 57	73	30	200	1037	57
MT 50, 51	63	31	106	1253	89
MT 63, 64	123	15	76	1773	tr.
MT 61, 62	111	34	98	2043	205
MT 73, 74	178	18	101	1480	207
MT 81, 82	62	25	>200	907	144
MT 75, 76	73	30	104	1475	209
MT 89, 90	73	30	97	1090	213
MT 108	63	18	78	827	174
MT 87, 88	50	22	>200	1653	195
MT 58	85	30	>200	1300	160
MT 65, 66	54	19	>200	930	47
MT 54	152	12	86	1480	tr.

NOTE: tr. = trace, less than 5 p.p.m.

TABLE V

Leukemia P-1534
(Average values, in p.p.m., for individual sample)

Sample	Element						
	B	Mn	Cu	Ag	Zn	Ni	Cr
LK 14	tr.	30	>200	tr.	433	tr.	tr.
LK 3	tr.	11	>200	tr.	330	tr.	37
LK 13	6	13	64	tr.	453	tr.	tr.
LK 2	tr.	18	131	tr.	797	tr.	53
LK 7	tr.	24	58	tr.	390	tr.	500
LK 1	12	9	>200	tr.	617	tr.	62
LK 12	tr.	13	>200	tr.	450	tr.	53
LK 5	tr.	10	65	tr.	380	tr.	68
LK 4	9	28	>200	tr.	653	tr.	tr.
LK 8	tr.	16	51	tr.	250	tr.	82
LK 6	tr.	26	78	tr.	550	n.d.	46
LK 11	tr.	13	>200	tr.	433	tr.	27
LK 9	tr.	14	61	tr.	520	tr.	36
LK 10	tr.	16	>200	tr.	480	tr.	tr.

NOTE: n.d. = not detected; tr. = trace, less than 5 p.p.m.

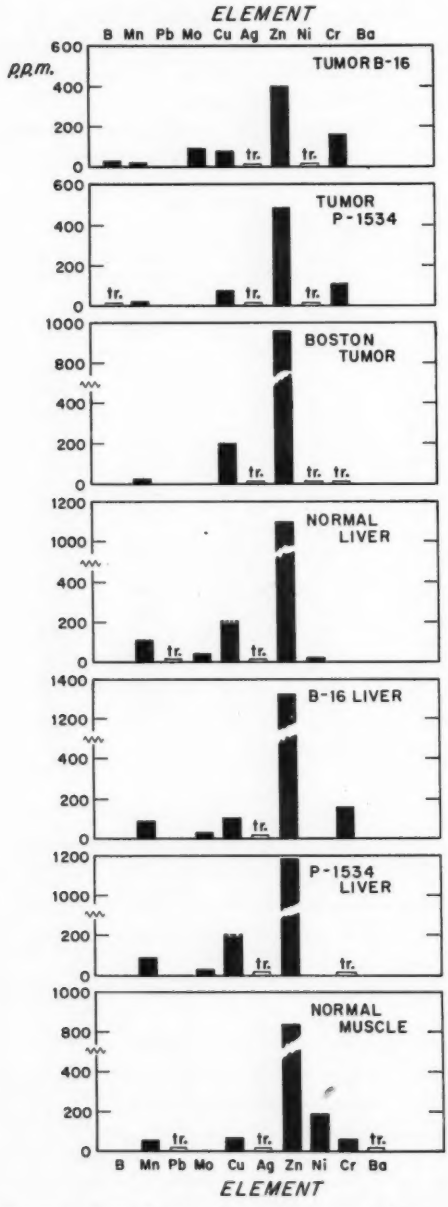

FIG. 1. Concentrations of trace elements in normal tissues and tumors.

which was much higher. Trace quantities were found in all three tumors, while tumor-bearing liver concentrations were below the limits of detection.

Chromium.—Similar high concentrations of Cr, approximately 150 p.p.m., were found in melanoma and in liver of melanoma-bearing animals, with wide variations within the sample population. No Cr was detected in normal liver, while normal muscle and P-1534 leukemia contained comparable concentrations, about 50 p.p.m., and the hamster sarcoma showed only trace amounts.

TABLE VI

Liver from leukemia-bearing hosts
(Average values, in p.p.m., for individual sample)

Sample	Element					
	Mn	Mo	Cu	Ag	Zn	Cr
LK 8	73	19	>200	tr.	1000	tr.
LK 11	109	20	>200	tr.	977	tr.
LK 4	93	24	>200	tr.	1099	tr.
LK 3	68	18	>200	tr.	1103	tr.
LK 9	111	26	>200	tr.	1249	tr.
LK 12	95	16	>200	tr.	1133	tr.
LK 1	87	28	>200	tr.	1450	tr.
LK 6	76	28	>200	tr.	1400	tr.
LK 2	58	21	>200	tr.	1365	tr.

NOTE: tr. = trace, less than 5 p.p.m.

TABLE VII

Hamster sarcoma tumor
(Average values, in p.p.m., for individual sample)

Sample	Element					
	Mn	Cu	Ag	Zn	Ni	Cr
BU 1	23	>200	tr.	1013	tr.	tr.
BU 2	26	>200	tr.	800	tr.	87
BU 3	16	>200	tr.	753	tr.	tr.
BU 4	28	>200	tr.	1036	tr.	tr.
BU 5	25	>200	tr.	1057	tr.	tr.

NOTE: tr. = trace, less than 5 p.p.m.

TABLE VIII

Average values, in p.p.m., of concentration
of trace elements in tissues

Tissue	Element									
	B	Mn	Pb	Mo	Cu	Ag	Zn	Ni	Cr	Ba
Normal muscle	n.d.	47	tr.	n.d.	64	tr.	893	180	54	tr.
Normal liver	n.d.	107	tr.	38	200	tr.	1095	15	n.d.	n.d.
B-16 liver	n.d.	84	n.d.	23	92	tr.	1338	n.d.	150*	n.d.
P-1534 liver	n.d.	86	n.d.	22	200	tr.	1182	n.d.	tr.	n.d.
Tumor B-16	21	15	n.d.	82*	69	tr.	399	tr.	144*	n.d.
Tumor P-1534	tr.	17	n.d.	n.d.	73*	tr.	481	tr.	52	n.d.
Boston tumor	n.d.	24	n.d.	n.d.	200	tr.	953	tr.	tr.	n.d.

NOTE: n.d. = not detected, tr. = trace, less than 5 p.p.m.
*Indicates wide range of variation in concentration among the sample population.

Discussion

This investigation has shown that each tumor examined differs, in trace metal composition, not only from the normal host tissue, but from other tumors as well. Each tumor has a characteristic profile of trace metal content. In the small sample of tumor types examined, it has not been possible to relate the distribution of trace metals to the proportion of stroma in the tumor or to the malignancy of the tumor. However, the measured differences in composition appear to be inherent in the tumors themselves, as the adequate number of tissue samples and the uniform conditions of diet, animal maintenance, and genetic background make chance effects unlikely.

The results of this study are not strictly comparable to those of other investigators, as other reports have been based on single human tumors, in which chance environmental effects may have been important, or on the normal tissues of animals, without comparable tumor analysis. However, some comparisons deserve discussion.

This analysis, on the whole, shows general agreement with previous reports of normal tissues by Fox and Ramage (5), by Webb (6), and by Sheldon and Ramage (7). Mn, Cu, and trace quantities of Ag were detected in all the tissues analyzed. Pb was found only sporadically. In all samples, Zn was found in the highest concentrations, possibly associated with carbonic anhydrase.

Our analysis of mouse tissue differs markedly from the results of Tietz *et al.* (8) on human tissues. In man, Tietz found Mo concentrations of 0 to 10 p.p.m. in normal subjects and considered higher concentrations abnormal. The livers of our normal mice had a mean Mo concentration of 38 p.p.m., with 23 and 22 p.p.m., respectively, in melanoma and leukemia host livers. In the tumors, Mo concentrations diverged from liver concentrations in opposite directions, with 82 p.p.m. in melanoma and no detectable Mo in the leukemia. With reference to Zn and Cu, our results are in agreement with those of Carruthers *et al.* (9), in that both metals were relatively low in concentration in the tumors, with the exception of Zn in the hamster sarcoma.

The melanoma and leukemia showed only half the Zn characteristic of the hamster sarcoma, and half the Cu. On the other hand, the sarcoma was much less rich in Cr than were the melanoma and leukemia. The melanoma was unique in its content of B and Mo, with B found in no other tissue, and Mo found in liver but not in the other tumors.

Such marked and characteristic differences in composition suggest that more extensive trace metal studies may be useful in several areas of oncology. The identification of certain metals with certain enzyme systems may facilitate study of the localization and function of the enzymes, particularly through use of radioisotopes of the associated metals. It may develop, with further study, that trace element profiles may be sufficiently characteristic to aid in the diagnosis of certain tumors, or in their treatment. Farr and associates (10) have used boron extensively in neutron capture therapy of brain tumors

and other elements may prove useful in other tumors. If the elements characteristic of certain tumors prove essential to the competitive advantage of the tumor cell, selective chelation of such elements by compounds such as the tetracyclines may become a practical therapeutic tool.

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SUGAR IN PULSES[1]

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Abstract

The occurrence of at least four non-reducing oligosaccharides has been observed in pulses. Three of the saccharides have been separated from resting seeds of green gram and have been characterized as raffinose, stachyose, and verbascose. A fairly similar pattern of composition of these sugars exists in a variety of pulses. The changes in the saccharides and the hydrolytic enzymes developed during germination have been studied.

Introduction

Pulses, defined as seeds of leguminous plants, provide a major portion of the protein requirement of the daily diet in India and for this reason the proteins of these seeds and their nutritive value have been the subject of much investigation. On the other hand, the sugars in pulses have been explored only to a limited extent. Thus, among the leguminous plant seeds, the carbohydrate constituents of soybean (sucrose, raffinose, and stachyose) have been studied by Tanret (1), Scholfield *et al.* (2), and Kawamura (3) and those of pea seedlings by Wanner (4). The sugars of cocoa beans have been studied by Cerbulis (5). The presence of the raffinose family of oligosaccharides in a variety of plant materials, including a few pulses, has been reviewed by French (6). However, no isolation or definite identification of these sugars from Indian pulses has been done so far. Courtois and his co-workers have described the presence of another series of saccharides in *Lychnis* (7, 8) containing varying numbers of galactose units together with one unit each of glucose and fructose. It is stated that these oligosaccharides have properties similar to the stachyose–verbascose series (9).

In this paper is reported the general occurrence of the galactose-containing saccharides, namely raffinose, stachyose, and verbascose, in a variety of pulses. The isolation and crystallization of raffinose and stachyose and the separation of verbascose from the resting seeds of one pulse, green gram (*Phaseolus radiatus*), provide definite identification of at least two of these sugars. The changes taking place in the sugar composition during germination of the seeds point to the importance of these saccharides in plant growth.

Experimental

The seeds, including the outer seed coat, of green gram (*Phaseolus radiatus*), bengal gram (*Cicer arietinum*), black gram (*Phaseolus mungo*), horse gram

[1]Manuscript received June 16, 1961.
Contribution from the Department of Biochemistry, Indian Institute of Science, Bangalore, India.
[2]Present address: Montreal Cancer Institute, Research Laboratories, Notre Dame Hospital, Montreal 24, Que.
[3]Deceased July 18, 1958.

(*Dolichos biflorus*), red gram (*Cajanus cajan*), lentil (*Lens esculenta*), cow pea (*Vigna sinensis*), and field beans (*Dolichos lablab*) used in the present investigation were obtained from the local grocer during the months of July–October. The reference sugars glucose, fructose, galactose, sucrose, and raffinose and the enzyme yeast invertase were obtained from the Nutritional Biochemicals Corp., Cleveland 28, Ohio. Almond emulsin was prepared according to Waksman and Davison (10).

Analytical Methods

Circular paper chromatography as described by Giri and Nigam (11) was used. The solvents for the separation of sugars were 1-butanol:acetone:water (2:7:1), 1-butanol:acetic acid:water (4:1:5), and 1-butanol:pyridine:water (6:4:3). The reagent aniline diphenylamine phosphate was employed (11) for the detection of all sugars; α-naphthylamine phosphate (11) for ketoses; and triphenyl tetrazolium chloride (11) for reducing sugars. Estimation of the sugars was carried out by eluting the sugar from the paper strips cut out from a well-developed chromatogram at the expected positions and by subsequently determining the ketose with Roe's resorcinol reagent (12) or reducing power with Nelson's arsenomolybdate colorimetric reagent (13). Reference curves were prepared with known amounts of glucose, fructose, galactose, sucrose, and raffinose, after identical treatment. Filter paper strips of areas equal to the test strips but containing no sugars provided the controls.

Preparation of the Extracts

The seeds were ground to pass through a 60-mesh sieve and a sample (0.5 g) of the powder was extracted with cold 70% aqueous ethanol (4.0 ml) for 12 hours with occasional shaking. After the extraction, the tubes were centrifuged and the supernatant stored for analysis.

The solutions of different pulses were spotted on a single chromatogram and the chromatogram developed with 1-butanol:pyridine:water, dried, and sprayed with aniline diphenylamine phosphate. It is observed that an identical pattern of free sugars exists for all pulses. Each of them exhibits four bands representing at least four different sugars. They are designated as 1, 2, 3, and 4, the fastest-moving compound being 1, followed by 2, 3, and 4 the slower-moving components. A comparison of these sugars with standard mixture of glucose, fructose, sucrose, raffinose, and stachyose run side by side showed no bands at the position of glucose and fructose: 1 corresponded with sucrose; 2, with raffinose; and 3, with stachyose in three solvent systems. A chromatogram cut into three sectors and sprayed with diphenyltetrazolium chloride and α-naphthylamine phosphate gave no bands with the first reagent and four bands with the second. It is obvious that the sugars are non-reducing ketosaccharides. The R_f values for the four oligosaccharides calculated from a circular paper chromatogram run in 1-butanol, pyridine, water solvent are 0.40, 0.26, 0.17, and 0.10 (the values for standard samples of glucose, fructose, sucrose, raffinose, and stachyose were found to be 0.48, 0.50, 0.40, 0.26, and 0.17, in the same solvent).

Separation of Oligosaccharides from Green Gram

Green gram powder (300 g) was extracted with cold 70% aqueous ethanol (1.5 liters) for 12 hours and subsequently the solution was filtered. The insoluble portion was re-extracted with 1.0 liter of 70% ethanol as before and the two filtrates combined and evaporated to a small volume *in vacuo*. Chromatographic analysis showed the presence of sucrose and three oligosaccharides whose R_f values were lower than that of sucrose.

The preliminary fractionation was carried out on a charcoal column. The concentrated solution (100 ml) was added on top of a charcoal–celite column prepared according to Whelan *et al.* (14). It was washed with 2 liters of water and then with 2 liters of 5% aqueous ethanol to remove inorganic impurities and oligosaccharide 1 (probably sucrose). Aliquots obtained after further elution with 5% ethanol were analyzed and they showed the complete removal of oligosaccharide 1. The rest of the oligosaccharides (2, 3, and 4) were then completely eluted from the column with 30% aqueous ethanol. A 4-liter solution of the saccharides resulted which was evaporated *in vacuo* to 10 ml. A light precipitate that formed was centrifuged out and the solution concentrated to a syrup (approx. 17 g). A portion of the syrup (800 mg) was then transferred to a column of washed Whatman 1 cellulose (3×30 cm). The column was irrigated with acetone:1-butanol:water (7:2:1), oligosaccharide 2 (120 mg) being contained in the eluate fraction, 500 ml–800 ml. The solvent composition was altered to 7:2:2, and the next 500 ml of eluate gave most of oligosaccharide 3 (230 mg). Continued elution yielded a mixture (85 mg) of oligosaccharides 3 and 4, and finally a fraction (250 mg) consisting of oligosaccharide 4 and a trace of oligosaccharide 3.

Raffinose

Oligosaccharide 2 (50 mg) crystallized when treated in the cold with a small quantity of acetic acid, and it was recrystallized from aqueous ethanol (yield, 10 mg), m.p. 87–90°, undepressed by admixture with raffinose hydrate. $[\alpha]_D^{28}$ +103.0° (*c*, 0.1, water).

Stachyose

Oligosaccharide 3 was purified according to French *et al.* (15), 100 mg of the syrup yielding 30 mg of crystalline material having a melting point of 155–160°, undepressed by admixture with stachyose tetrahydrate. $[\alpha]_D^{28}$ +131.0° (*c*, 0.35, water).

Oligosaccharide 4

The syrup was extracted several times with dry methanol and converted to a white powder. $[\alpha]_D^{28}$ +158.5° (*c*, 1.8, water). (Verbascose has $[\alpha]_D$ +170° (6).) The compound was characterized tentatively as verbascose, from the following properties:

(*a*) A positive Raybin test (16) was given.

(*b*) A linear plot obtained for log $R_f/1-R_f$ vs. number of hexose units, as described by French (6) for unidimensional paper chromatography, indicated

that oligosaccharide 4 is the pentasaccharide of the series containing oligosaccharides 1, 2, and 3.

(*c*) Partial hydrolysis of oligosaccharide 4 with mineral acid yeast invertase and almond emulsin yield various fragments (paper chromatographic evidence) —glucose, fructose, galactose, sucrose, melibiose, raffinose, stachyose—consistent with the structure of verbascose.

(*d*) The ratio fructose:glucose:galactose in the material was 1:1.07:3.03 (requires 1:1:3).

Composition of the Oligosaccharides in Pulses

Known amounts of ethanolic extract of sugars from various pulse powders were spotted at three adjacent positions on a paper chromatogram and the chromatogram developed with 1-butanol:pyridine:water solvent. The sugars were located in the middle sector by spraying the adjacent areas with aniline diphenylamine phosphate. The paper strip corresponding to each sugar was cut out from the middle sector and the sugar eluted in 2 ml of water. The amount of fructose in each saccharide was estimated with Roe's reagent. Since 1, 2, 3, and 4 are sucrose, raffinose, stachyose, and verbascose and each contains a single fructose unit the total sugar content was computed by multiplying the fructose value of sucrose by two, of raffinose by three, of stachyose by four, and of verbascose by five. The conditions of Roe's determination are such that total liberation of the fructose moiety takes place. Table I gives the amount of each saccharide in grams per 100 grams of seed powder. It can be observed that a fairly uniform pattern exists in all pulses. Thus, each pulse contains a larger amount of verbascose, stachyose, and less raffinose.

TABLE I

Sugar composition of pulses (grams sugar/100 grams seed)

Pulses	1 (sucrose)	2 (raffinose)	3 (stachyose)	4 (verbascose)
Green gram	1.8	0.8	2.5	3.8
Black gram	1.6	0.51	1.8	3.7
Red gram	1.6	1.1	2.7	4.1
Horse gram	2.7	0.7	2.0	3.1
Bengal gram	2.4	1.0	2.5	4.2
Lentil	2.1	0.6	2.2	3.0
Cow pea	1.5	0.4	2.0	3.1
Field bean	1.3	0.5	2.1	3.6

Sugars in Various Stages of Germination

To study the changes during germination, samples consisting of 1 g of green gram seeds were kept in 2 ml of distilled water at room temperature. After 6, 12, 24, and 48 hours of germination they were removed and kept in a vacuum desiccator. They were ground with 5 ml of 70% ethanol, the extract centrifuged, and the supernatant analyzed by quantitative chromatography as

described above. The results are presented in Fig. 1. It can be seen that during germination there is a liberation of fructose whose level rises as germination proceeds and at the same time there is a rapid removal of oligosaccharides.

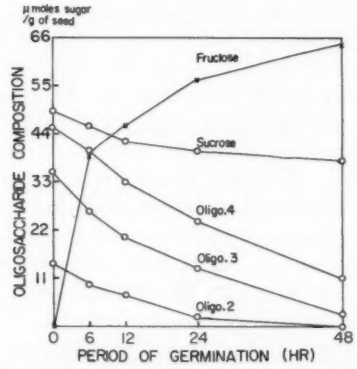

FIG. 1. Changes taking place in the sugar composition of green gram during germination: oligo. 2, raffinose; oligo. 3, stachyose; oligo. 4, verbascose.

Sucrose remains fairly constant. Probably the oligosaccharides themselves are first broken down to sucrose before being hydrolyzed to fructose, glucose, and galactose. The rapid transformation of these saccharides indicates their probable participation in the process of germination and plant growth. A control experiment in which the seeds were soaked in a larger amount of water failed to show any leakage of the sugars on chromatography of the surrounding fluid after 24 and 48 hours of germination.

Enzyme Activity of the Seeds During Germination

Resting and germinated seeds at various stages were examined for enzymes that hydrolyze disaccharides. The activity of α-glucosidase was tested with maltose as substrate, β-glucosidase with cellobiose, α-galactosidase with melibiose, β-galactosidase with lactose, and invertase with sucrose and raffinose. In each case 50 mg of the substrate (disaccharides and raffinose) was incubated with 1 ml of the dialyzed green gram extract (5 g in 20 ml distilled water) for 24 hours at 37° C under a layer of toluene and after heat in activation the digests were analyzed by paper chromatography for the formation of monosaccharides. It was observed that the resting seeds had very little hydrolytic activity against the substrates tested except melibiose, which was hydrolyzed to glucose and galactose to a small extent. After germination for 3 days all the hydrolases were elaborated. Significant hydrolysis was observed for sucrose, raffinose, and maltose and less for cellobiose and lactose. During maltose hydrolysis there occurred a simultaneous formation of higher saccharides by transglucosidation (17).

Discussion

It is observed that the carbohydrates of pulses when subjected to paper chromatography appear as a regular series of bands with decreasing R_f values from that of sucrose. The chromatographic pattern of all pulses examined is found to be the same. These carbohydrates are non-reducing and yield glucose, galactose, and fructose on hydrolysis. The galactose content of these oligosaccharides increases in the order of 1, 2, and 3. The oligosaccharides from one of the pulses (*Phaseolus radiatus*) were separated by chromatography on cellulose and characterized by their optical rotation, paper gram mobility, color reactions on paper, determination of the end products of total, partial, and enzymic hydrolysis and by diazouracil test, as raffinose, stachyose, and verbascose. The first two sugars were also obtained in a crystalline form. Thus each component is a derivative of raffinose and the series is probably formed by the successive addition of galactose residues to sucrose. The determination of each component in various pulses shows uniformity. Each one contains a large amount of verbascose followed by stachyose and sucrose and relatively smaller amount of raffinose. The over-all composition of these saccharides is approximately 10% of the dry seeds. The raffinose family of oligosaccharides seems quantitatively more important than the polysaccharide, which occurs in the pulses in a much lower amount.

A study of the germination of the seeds shows another important aspect of the saccharides. Thus within a few hours of soaking the seeds in water there occurs a decrease in the amount of raffinose, stachyose, and verbascose and in 48 hours the first two completely disappear. Unlike the composition of stachyose and verbascose, that of sucrose remains fairly constant during this period of germination. Fructose appears and increases with time. However, no balance can be observed with the disappearance of the oligosaccharides and the formation of sucrose and other monosaccharides. The phenomenon is to be regarded as a result of α-galactosidase activity of the germinating seed which hydrolyzes the oligosaccharides to monosaccharides and sucrose. Glucose, fructose, and to a large extent galactose play an important function in plant growth and the synthesis of cellulose and stem proteins.

The difference in carbohydrase activity between resting and germinating seeds can be attributed to the difference in the enzyme make-up of embryo and endosperm separately (18). The formation of these oligosaccharides must be due to specific enzymic systems operative in the seed during the ripening of the grain. The role of D-galactose 1-phosphate or uridine-diphosphogalactose may be important in bringing about their synthesis. But so far the expectation has not been realized.

Acknowledgments

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THE INFLUENCE OF DIETARY FAT ON THE FATTY ACID COMPOSITION OF LIVER, CARCASS, AND MILK OF RATS[1]

JOYCE L. BEARE

Abstract

Fatty acids of liver, carcass, and milk of rats fed corn oil, rapeseed oil, partially hydrogenated herring oil, or margarine were examined by gas–liquid chromatography. Appreciable quantities of linoleic acid were maintained in the tissues and milk, even when the hydrogenated herring oil with a low level of linoleic acid was fed. The proportion of C_{20} and C_{22} acids deposited or secreted was related to that of the diet, and was highest with rapeseed oil. In the livers of rats fed each diet, long-chain, polyunsaturated acids were observed. The fatty acids of milk more closely reflected the dietary pattern than did those of the tissues.

Introduction

Since rapeseed oil in the diet was found to depress appetite (1, 2) and to change the fatty acid composition of the tissues of rats (3), it was decided to examine the effect of other dietary fats containing long-chain fatty acids. Hydrogenated herring oil and a margarine containing marine oil were compared with corn oil and rapeseed oil during periods when the rats grew rapidly or nursed their young. By means of gas–liquid chromatography, the influence of these dietary fats on liver, carcass, and milk fatty acids was investigated.

Method

A purified diet (2) containing 20% by weight of corn oil, rapeseed oil, hydrogenated herring oil, or a margarine possessing some marine oil was fed ad libitum to male, weaning, Wistar rats of the Food and Drug colony. Ten animals per group, randomized within blocks of individual cages according to initial weights, were maintained on their respective regimens for 6 weeks. At the termination of this period, the rats were anesthetized with ether, bled, and the liver and adrenals removed and weighed. The Abell method (4) was used to determine the serum cholesterol levels.

Lipid samples were obtained from livers and ground degutted carcasses by the extraction method of Bligh and Dyer (5), methylated, and subjected to gas chromatography as previously described (6).

To obtain milk samples, seven mothers were fed each of the diets employed in the first study for 14 to 15 days following parturition. The stomach contents of the young rats were removed and the fatty acids analyzed as in the previous study (6).

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

Results and Discussion

A chromatogram of each of the dietary fats is shown in Fig. 1. Their chief characteristics are: the high concentration of C_{18} acids, particularly of linoleic acid, in corn oil; the high levels of eicosenoic and erucic acids in rapeseed oil; the low content of linoleic acid, the odd-numbered fatty acids, and the series of C_{20} acids in partially hydrogenated herring oil; the apparent mixture of vegetable and marine oils with prominent C_{16}, C_{18}, and C_{20} acids in margarine.

From Table I it is seen that rapeseed oil was associated with a decreased food intake, whereas the other dietary oils were not. The total C_{20} and C_{22} acids in rapeseed oil, hydrogenated herring oil, and margarine varied in the ratio of

TABLE I

Food intake, weight gain, liver and adrenal weight, and serum cholesterol level of rats fed corn oil (CO), rapeseed oil (RSO), hydrogenated herring oil (HH), or margarine (M) for 6 weeks (10 rats per group)

Dietary fat	Food intake (g)	Wt. gain (g)	Adj. wt. gain* (g)	Liver wt. (g)	Adrenal wt. (mg/pair)	Serum cholesterol (mg/100 ml)
CO	339±8†	134±3	130	6.96±0.20	26.9±1.1	97.8±5.8
RSO	253±5	88±2	119	6.18±0.21	27.9±1.6	71.3±1.6
HH	398±16	144±7	118	8.57±0.81	26.5±1.2	62.0±3.8
M	338±6	114±3	111	6.93±0.13	27.6±1.0	62.7±2.7

*Weight gain adjusted for food consumption.
†Mean±S.E.

4:2:1. Since a diet containing 10% instead of 20% rapeseed oil did not depress appetite (7), it was assumed that the other fats did not have the same effect as rapeseed oil because of their lower concentration of long-chain fatty acids. The rats receiving the hydrogenated herring oil consumed more food than did the other groups, and also gained more weight. When all weight gains were adjusted for food consumption by covariance analysis, the diet containing corn oil appeared to be the most efficiently utilized, and the diet containing margarine the least.

The livers of rats fed hydrogenated herring oil were significantly heavier than those of other rats which did not differ from each other. There were no significant differences in adrenal weights. As found previously in rats (8), the highest values for serum cholesterol were obtained in the animals fed corn oil.

In Fig. 2 are shown typical chromatograms of esters of fatty acids of liver tissue. The results of fatty acid analyses of individual livers and carcasses and their standard deviations are shown in Table II. Dietary fatty acids had more influence on the carcass fat than on the liver fat where a large proportion of the fatty acids are constituents of phospholipids which have a relatively constant composition.* The proportion of myristic acid was higher in hydrogenated herring oil and margarine and in the carcass fats of the rats fed those

*B. M. Craig, C. G. Youngs, J. L. Beare, and J. A. Campbell. Unpublished data.

FIG. 1. Chromatograms of methyl esters of fatty acids of corn oil (CO), rapeseed oil (RSO), partially hydrogenated herring oil (HH), and margarine (M).

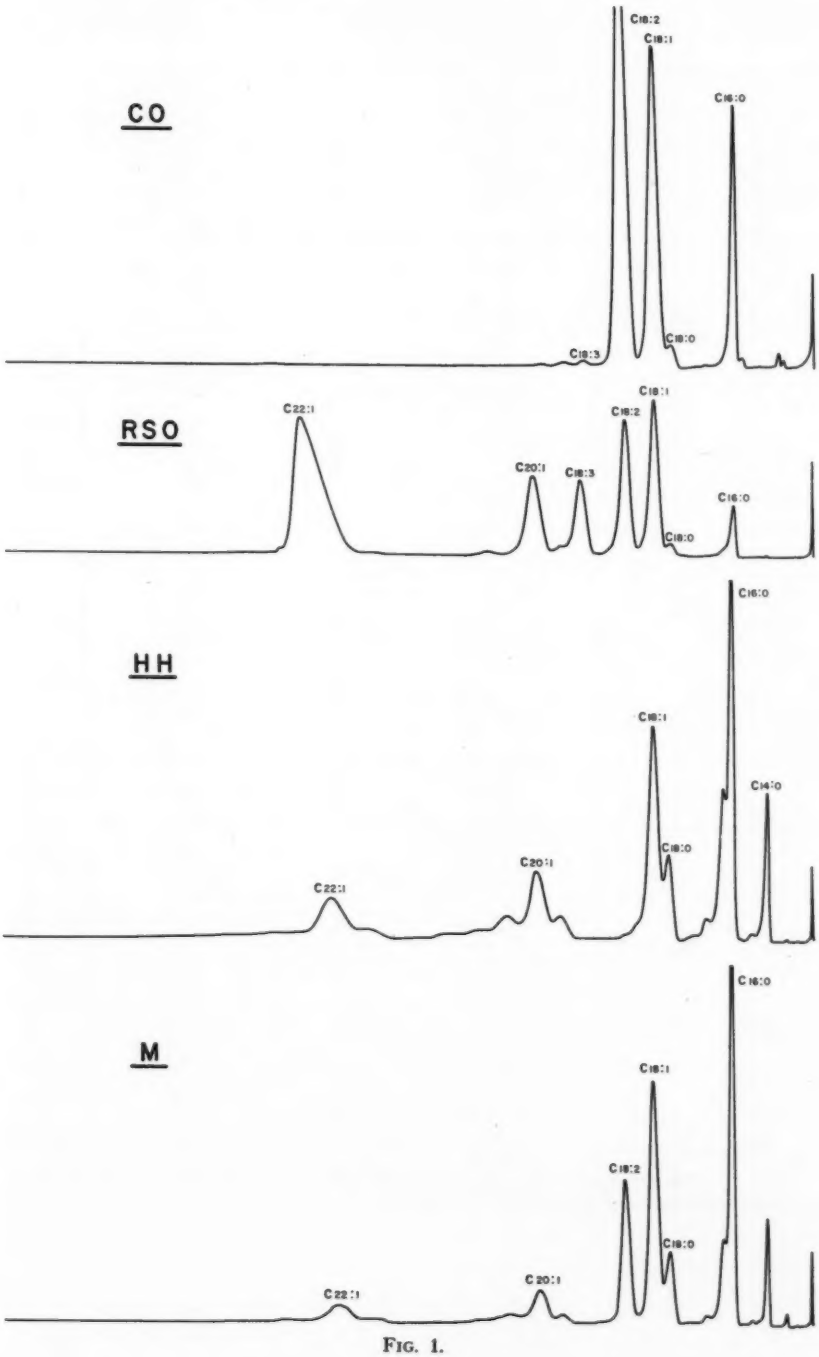
CO
C18:2
C18:1
C16:0
C18:3
C18:0
RSO
C22:1
C18:1
C18:2
C20:1
C18:3
C16:0
C18:0
HH
C16:0
C18:1
C14:0
C18:0
C20:1
C22:1
M
C16:0
C18:1
C18:2
C18:0
C20:1
C22:1

FIG. 1.

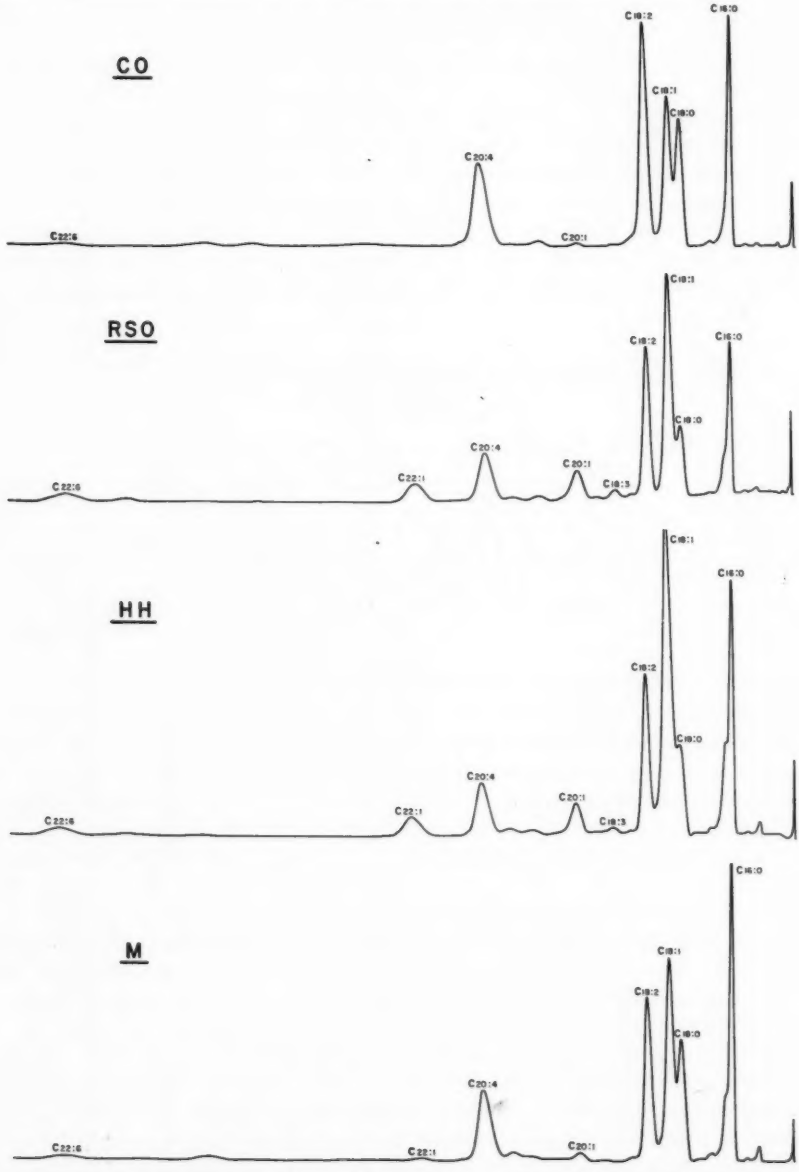

FIG. 2. Chromatograms of methyl esters of livers of rats fed corn oil, rapeseed oil, partially hydrogenated herring oil, or margarine.

TABLE II

Fatty acids of diet, liver, and carcass

Fatty acid	Source	% of total fatty acids			
		CO	RSO	HH	M
$C_{14:0}$	Diet	0.3	0.1	5.0	3.4
	Liver	0.2±0.1	3.7±0.4	0.8±0.2	0.7±0.3
	Carcass	0.9±0.2	1.2±0.3	4.6±0.7	3.9±0.8
$C_{15:0}$	Diet			0.4	0.2
	Liver	0.1±0.0	0.2±0.1	0.2±0.1	0.2±0.1
	Carcass	0.1±0.1	0.1±0.1	0.5±0.2	0.3±0.2
$C_{16:0}$	Diet	12.5	2.7	26.8	35.2
	Liver	16.0±1.4	8.8±2.2	14.4±1.4	18.5±2.2
	Carcass	13.5±1.7	10.9±1.7	19.3±3.9	20.9±2.6
$C_{16:1}$	Diet		1.1	13.1	8.0
	Liver		3.8±1.1	7.9±1.3	5.2±1.3
	Carcass	2.5±0.5	5.0±0.8	11.7±2.8	14.5±2.0
$C_{17:0}$	Diet			1.9	0.7
	Liver	0.6±0.7	0.1±0.1	0.9±0.8	0.5±0.1
	Carcass	0.2±0.1		0.9±0.4	1.0±0.4
$C_{17:1}$	Diet			0.3	0.3
	Liver				
	Carcass			0.4±0.3	0.3±0.2
$C_{18:0}$	Diet	2.1	1.4	6.0	6.5
	Liver	15.1±0.4	7.7±2.5	11.1±2.0	14.9±1.6
	Carcass	3.1±0.6	1.5±0.6	3.4±0.8	2.8±0.8
$C_{18:1}$	Diet	28.3	15.1	16.8	20.9
	Liver	19.7±3.2	34.2±5.8	27.6±1.1	20.9±5.0
	Carcass	35.5±1.1	34.7±2.4	36.4±2.4	36.0±1.9
$C_{18:2}$	Diet	54.2	13.9	0.5	11.8
	Liver	25.2±1.7	17.8±1.9	17.4±2.4	18.0±1.5
	Carcass	42.6±2.2	16.4±1.1	13.4±2.0	12.5±1.7
$C_{18:}$*	Liver	0.2±0.3	0.1±0.2	0.2±0.3	
$C_{18:3}$	Diet	2.0	9.4		
	Liver	0.4±0.4	1.2±0.3	0.3±0.3	0.4±0.4
	Carcass	0.8±0.3	5.2±0.7	0.8±1.5	0.2±0.2
$C_{20:0}$	Diet	0.4		3.0	
	Liver	0.1±0.1	0.1±0.2	0.1±0.2	1.1
	Carcass			0.6±0.2	0.8±0.4
$C_{20:1}$	Diet	0.2	12.7	9.0	4.7
	Liver	0.8±0.4	6.7±1.8	2.0±1.4	1.1±0.3
	Carcass	0.6±0.2	10.2±0.8	4.4±0.7	3.9±0.6
$C_{20:2}$	Diet		0.7	4.2	2.1
	Liver	0.5±0.5	0.3±0.4	0.1±0.2	0.5±0.3
	Carcass	0.1±0.1	0.8±1.1	1.1±0.3	0.9±0.3
$C_{20:}$*	Liver		0.1±0.2	0.8±0.5	0.5±0.3
$C_{20:3}$	Diet			1.6	
	Liver	0.2±0.3	0.3±0.4	1.6±1.1	2.0±0.5
$C_{20:4}$	Diet			0.4	
	Liver	17.5±1.9	8.1±2.1	10.6±0.6	14.2±1.5
	Carcass	2.0±0.8	1.0±0.5	1.1±0.3	1.0±0.5

*Unknown.

TABLE II (*Concluded*)

Fatty acids of diet, liver, and carcass

Fatty acid	Source	% of total fatty acids			
		CO	RSO	HH	M
$C_{22:0}$	Diet			1.6	0.7
$C_{22:1}$	Diet		42.9	8.6	3.7
	Liver	0.3±0.6	5.6±1.6	1.5±1.3	0.3±0.3
	Carcass	0.1±0.2	11.3±0.9	0.8±0.5	0.7±0.6
$C_{22:2}$	Diet		0.4		
	Carcass		1.4±0.5		
$C_{22:3}$	Liver	0.7±0.5	0.2±0.5		
$C_{22:4}$	Liver	0.9±0.6	0.2±0.5	0.8±0.7	1.0±0.6
$C_{22:5}$	Liver	0.2±0.5	0.9±1.1	0.2±0.2	
$C_{22:6}$	Liver	1.2±0.8	3.0±0.8	1.5±0.5	1.5±0.5

*Unknown.

oils than in corn oil or rapeseed oil or the rats fed those oils. As expected from previous work with animal fats (9, 10, 11), oils of marine origin and rat liver and carcass fats contained pentadecanoic acid. The levels of palmitic and palmitoleic acids in the diet influenced the tissue levels of those constituents. Heptadecanoic acid occurred in herring oil and margarine, in carcasses of the rats fed those fats, and in some livers of each group. Heptadecenoic acid, found in musk-ox fat by Chisholm and Hopkins (12), might have been present, but the chromatogram peak in question might also be identified as isooctadecanoic acid (13). Liver lipids contained relatively high proportions of stearic acid (14), mostly in the phospholipid fraction (15). Although the dietary level of oleic acid varied from 15.1 to 28.3% the tissue levels appeared to be independent. The high level of linoleic acid in corn oil increased the content of that acid in tissue fat, particularly in the carcass. As shown in the depot fat of mice by Tove and Smith (16), and of rats by Mead (17), linoleic acid was inversely related to palmitoleic acid, but linolenic acid did not increase with linoleic acid. Despite the small quantity of dietary linoleic acid available to rats fed the hydrogenated herring oil, the level of that acid in the body fat was similar to that of rats fed rapeseed oil or margarine. This finding could be indicative of a preferential retention of the sparsely available linoleic acid. In some livers there appeared to be an isomer of the common linoleic acid which emerged immediately after it from the chromatographic column, as was observed previously (8). Rats fed rapeseed oil which contained 9.4% linolenic acid had appreciably more of that acid in their tissues than other rats.

The combined C_{20} and C_{22} fatty acids in the diet and in the carcass of rats fed corn oil was 0.6 and 2.8% respectively; rapeseed oil, 56.7 and 24.7%; hydrogenated herring oil, 28.4 and 8.0%; margarine, 11.2 and 7.3%. Small amounts of arachidic acid in the diet had little influence on its deposition in the animals, whereas eicosenoic acid was readily deposited, as shown by

Hopkins *et al.* (18), and reflected the dietary levels. Eicosadienoic acid was found in some of the livers and particularly in the carcasses of those animals supplied with it. In the livers there were also small amounts of polyunsaturated C_{20} acids and an eicosatrienoic acid, observed in beef liver by Klenk and Montag (19). Arachidonic acid, which was synthesized from linoleic acid (20, 21), was found to be most pronounced in the rats fed corn oil, and to be present in lower and similar concentrations in the other animals. Although linolenic acid was believed to be converted to eicosapentaenoic acid (22), such an acid was not detected on the chromatograms. The dietary behenic acid seemed not to be in the rat tissue. Of particular interest was the tissue erucic acid, which amounted to 11.3% of the carcass fatty acids in rats fed rapeseed oil, and was about 26% as concentrated as in the diet. The small amount of docosadienoic acid of rapeseed oil appeared to be easily retained by the rat. On the basis of relative retention times on the chromatographic column, the liver was found to contain C_{22} acids with three, four, five, and six double bonds, the last being the most pronounced. By studying the oxidative degradation products of liver fatty acids of rats fed labelled acetate, Klenk (21, 23) identified these highly unsaturated, long-chain fatty acids which originated from dietary linoleic and linolenic acids.

Table III gives the data of the young rats from which the stomach contents were obtained. One rat fed margarine failed to produce a litter, and two others on the same diet and one fed hydrogenated herring oil ate their offspring. The

TABLE III

Litters nursed by rats fed different dietary oils

Dietary fat	No. of litters	No. of young per litter	Litter wt. (g)	Mean wt. of young (g)
CO	7	10.6±0.5	252±12	24.1±1.5
RSO	7	9.7±0.6	200±8	21.0±1.2
HH	6	8.8±0.6	169±8	19.3±0.7
M	4	9.5±0.8	220±11	23.2±0.8

weights of the 14- to 15-day-old litters of mothers supplied with corn oil were significantly greater ($P = 0.01$) than of those receiving rapeseed oil or hydrogenated herring oil. Rats fed margarine had significantly heavier litters than those fed hydrogenated herring oil.

In Fig. 3 the values for the milk fatty acids with their standard deviations are shown beside the dietary levels of fatty acids. The proportion of myristic in the total fatty acids was greater in milk than in the tissues, but was again influenced by the dietary oil. The quantities of palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, eicosenoic, eicosadienoic, and erucic acids reflected the dietary pattern of fatty acids, generally to a greater extent than the carcass fatty acids. Arachidonic acid, not provided in the diet, was related to the varying amounts of dietary linoleic acid. Within the pattern of fatty acids

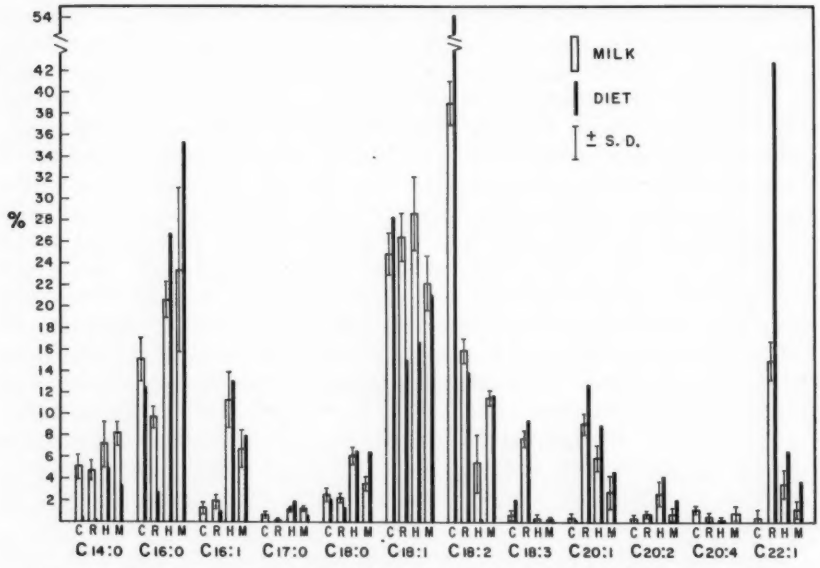

FIG. 3. The percentage of fatty acids in the total fatty acids of milk and diet of seven rats fed corn oil (C), seven rats fed rapeseed oil (R), six rats fed hydrogenated herring oil (H), and four rats fed margarine (M).

secreted by the rat, appreciable variations could be imposed by changes in the dietary fat.

It was of interest that the rats which had been fed erucic acid for several generations (6) secreted milk containing somewhat less than half of the amount of that fatty acid produced by these rats which had received rapeseed oil only while nursing the young. From these findings, it appeared that over long periods of time there was some adaptation in the utilization of erucic acid.

The effect of long-chain fatty acids on food intake and the composition of fatty acids deposited or secreted by the rat appeared to be related to the quantities of such acids present in the diet. Milk showed the best reflection of the dietary pattern of fatty acids.

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SEASONAL ADJUSTMENTS IN CAPTURED WILD NORWAY RATS

I. ORGAN WEIGHTS, EAR VASCULARIZATION, AND HISTOLOGY OF EPIDERMIS[1]

O. HÉROUX

With the technical assistance of DONNA WRIGHT

Abstract

Male wild rats (*Rattus Norwegicus*) captured during the winter had different organ weights from similar animals captured during the summer. These seasonal changes in organ weights were different from those observed in white rats acclimated to cold in a cold room (indoor) or acclimatized to winter outdoors in groups of 10 to a cage.

Unlike both the indoor and outdoor cold-conditioned white rats, the wild rats, in winter, did not have a reduced muscle mass as compared to the summer-captured rats, or a reduced abdominal fat reserve, or an enlargement of the liver and kidney. The abdominal fat reserve actually increased during the winter.

Like the outdoor white rats, the wild rats, during the winter, did not show the enlargement of the thyroids, adrenals, pituitary, and digestive tract or the reduction in pelt and the ear cold injury that were invariable outcomes of continuous individual exposure to a constant low-temperature indoors. Like the indoor and unlike the outdoor white rats, however, they showed an hypertrophy of the heart and an increased vascularization of the ear.

Because the wild rats trapped during the winter showed very few of the morphological changes usually found in cold-exposed white rats, it is suggested that winter conditions encountered were not stressful to the wild rats.

Introduction

In a previous series of papers (1–4) morphological adjustments to low temperature were compared in white rats exposed either to fluctuating environmental conditions prevailing outdoors during the winter or to indoor constant-temperature conditions.

Under these two different types of cold exposure, the rats were observed to have a reduced muscle mass, larger livers and kidneys, and smaller genitals than the controls. The enlargement of the adrenals, thyroids, pituitary, heart, and digestive tract and the reduction of the pelt weight and of the mesenteric and subcutaneous fat, which have repeatedly been found in indoor cold-acclimated rats, did not take place in outdoor winter rats. Moreover, increased vascularization of the ears, greater thickness of the epidermis, and cold injuries usually found in ears of indoor cold-acclimated rats were not present in outdoor winter rats.

Whereas the outdoor white rats were exposed to the prevailing environmental conditions, they were not running free in the field but kept in captivity, 10 per cage. The conditions under which these white rats were studied merely approached the natural conditions under which wild animals live. It remained

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to be shown whether seasonal acclimatization would be similar in closely related wild Norway rats living free in their natural habitat.

This paper, the first of a series, will describe the seasonal adjustments in the weights of the different organs, the vascularization of the ear, and the histology of the ear skin.

Experimental Procedures

During the winter 1955–56, and the following summer 1956, wild rats (*Rattus Norwegicus*) of both sexes were captured on a farm at Orleans near Ottawa, Ontario. Since in previous studies on white rats, capillary counts and organ weights were obtained only on male animals, in order to be able to make proper comparison between white and wild rats, this study was limited to male wild rats.

Capillaries were counted, and thickness of epidermis was measured, on longitudinal sections of the ears cut at 10 μ and stained with ammonium molybdate benzidine for capillaries and haematoxylin eosin for epidermis. The capillary counts were made according to a method described in a previous paper (5). Thickness of epidermis was measured at 15 locations chosen at random on each side of the ear sections. An average of those 30 measurements was then corrected for shrinkage according to the procedure described in reference 5.

During the summer 1958 and 1959 and the winter 1958–59, wild rats were captured on a dump at Kingston, Ontario. The rats were anaesthetized with chloroform and bled not more than 2 hours after capture. The dissection and weighing of the organs were carried out immediately according to the methods described in a previous paper (6).

The summer rats were captured in July and the winter rats in February. The average temperature for the month of July was 20° C in Ottawa and 21° in Kingston; in February it was −8° C in Ottawa and −7° C in Kingston.

Results

Body Weight

The body weights of the 12 winter rats ranged from 277 to 430 g with an average of 348 ± 15 g. This is slightly, but not significantly, more than the average of 335 ± 35 g (ranging from 188 to 526 g) for the 10 summer rats. Thus the two seasonal groups can be regarded as comparable as far as the sizes of the individual animals are concerned.

Organ Weight in Relation to Body Weight

In contemplating organ weights in variable groups of animals, we are plainly obliged to take individual body-weight differences into account. At first sight the solution is to transform the absolute organ weights into relative weights, e.g. to express them as weight per 100 grams of body weight. But it has been argued elsewhere (6) that this widely followed practice, which is so simple and which appears so unobjectionable, is in fact difficult to justify. It rests on the

assumption that the weight of any given organ is normally proportional to body weight. This is not really a sound expectation, especially when age, as well as size, is a variable, because the parts of an organism do not develop at the same rate. It is well known that the proportions change as maturity approaches. It seems best, then, not to make any assumptions about the relation of the parts to the whole, but to estimate the relation from the experimental data themselves. This can most readily be done by way of the flexible allometric relation,

$$Y = cX^b$$
$$\equiv \log Y = a + b \log X,$$

where Y is the weight of the organ and X is body weight. The key parameter is b, easily estimated from the raw data as the regression coefficient in the second, logarithmic form, of the above relation, by the method of least squares. It is often found, in work of this kind, that b is approximately the same for different treatment groups, and this is so in the present instance. (Incidentally, the common assumption of proportionality between X and Y is equivalent to the assumption that b is unity; and this assumption can itself be statistically tested).

The pooled least-squares estimate of b can be used to make proper allowance for the body-weight differences, to yield, in other words, an "adjusted" organ weight (again in terms of logs), by appropriate application of the equation given above. For further details, including estimation of error, any standard statistical text should be consulted.

In logarithmic units, differences in thymus and thyroid weights were not significantly correlated with differences in body weight. Compared therefore on an absolute basis, thyroid and thymus weights of winter rats were, on the average, greater than those of summer rats but the differences were not statistically significant (Table I).

All other individual organ weights were significantly correlated with body weight. Corresponding regression coefficients, specifying the average increment in log organ weights per unit increment in log body weight, did not differ significantly from summer and for winter animals. The coefficients for digestive tract, fat, adrenals, and pituitaries all differed significantly from unity, indicating disproportionalities in the observed weights of these organs in large and small individuals. On the average, large animals had proportionately smaller digestive tracts and pituitaries and proportionately more fat than small animals in both summer and winter (Table I). None of the other regression coefficients was demonstrably significantly different from unity. Some, however, were rather imprecisely determined (Table I), providing no clear indication of direct proportionality or disproportionality.

Of the 13 organ weights correlated with body weight only four differed perceptibly in the two groups. Heart and fat were, on the average, heavier in winter animals (1% level of significance), genitals, and digestive tract content were heavier in summer animals (1% and 5% level respectively).

TABLE I

Organ weights, Kingston wild rats

Organ	Observed mean weights		Regression coefficient (b) of log weights on log body weights	Adjusted mean weights (after allowance, through b for body-weight differences)		The ratio Y_s/Y_w	"t" $Y_s - Y_w$ $N = 18$
	Summer (Y_s)	Winter (Y_w)		Summer (Y_s)	Winter (Y_w)		
Fat	11.243	17.666	1.588 ± 0.199	12.00	16.71	0.72	3.43**
Soleus	0.15305	0.16924	1.123 ± 0.210	0.1603	0.1629	0.98	−0.206
Heart	1.3827	1.652	1.101 ± 0.117	1.3213	1.5923	0.83	−3.24**
Kidney	3.313	3.396	1.094 ± 0.123	3.467	3.2735	1.06	0.96
Liver	16.540	16.958	1.094 ± 0.116	17.30	16.34	1.06	1.042
Pelt (skin)	42.308	48.778	1.003 ± 0.100	44.10	47.13	0.94	−1.93
Digestive tract content	17.730	11.887	0.991 ± 0.381	18.75	11.48	1.61	2.61*
Lung	3.3166	3.3544	0.913 ± 0.232	3.443	3.251	1.06	0.510
Genitals	11.367	10.069	0.823 ± 0.097	11.75	9.79	1.20	3.76**
Carcass	163.69	185.96	0.796 ± 0.11	169.13	180.97	0.93	−1.70
Spleen	1.495	1.928	0.722 ± 0.223	1.503	1.879	0.80	−1.94
Intestinal tract and pancreas	13.749	15.260	0.718 ± 0.134	14.16	14.89	0.95	−0.78
Pituitary	0.01040	0.9570	0.687 ± 0.125	0.0099	0.0794	0.12	−0.50
Adrenals	0.12921	0.10520	0.569 ± 0.23	0.0792	0.0933	0.85	−1.48
Brain	1.9598	1.8755	0.272 ± 0.062	1.9820	1.8583	1.07	2.17*
Thymus	0.0710	0.0838	—	—	—	—	−0.57
Thyroid	0.0308	0.0345	—	—	—	—	−1.25

**Values significant at 1% level.
*Values significant at 5% level.

Ear Histology

A greater number of capillaries/mm² were open in the ears of winter rats than in the ears of summer rats but there was no difference in the thickness of ear epidermis between the two groups (Table II). There were no macroscopic or microscopic signs of cold injury in any of the captured winter rats.

TABLE II

Number of capillaries/mm² and thickness of epidermis in ears of summer and winter wild rats

	Summer	Winter	Difference
No. of capillaries/mm²	45.5±9.7†(7)	73.4±8.0(11)	28.0±12.6*
Thickness of epidermis (μ)	24.7±4.9 (7)	24.1±3.6(11)	0.6± 6.8

NOTE: Number of animals in parentheses.
* = statistically significant at 5% level.
† = standard error.

Discussion

The morphological adjustments to the winter conditions found in captured wild rats are evidently different from the ones found in white rats that are cold-acclimated indoors under constant low-temperature conditions or outdoors when kept in groups of 10 in a cage exposed to the outside fluctuating environmental conditions during the winter (1–4).

Unlike both the indoor and outdoor white rats, the wild rats in winter do not have a reduced muscle mass, as shown by the normal carcass weight, a reduced deposition of abdominal fat, or an enlargement of the liver and the kidney (Table I). In fact, the abdominal fat reserve is increased during the winter. A similar phenomenon has been observed in winter-captured mice (7) as well as in birds (8). It is not clear why the white rats react to cold indoors and to the seasonal factors outdoors by reducing their fat reserves whereas the wild rats do exactly the reverse. It is true that in the outdoor white rats the reduction in abdominal fat was not quite statistically significant but it was certainly not increased during the winter as found in the wild rats.

Unlike cold-acclimated rats, wild winter rats like white rats exposed to winter conditions did not show any significant enlargement of the thyroids, adrenals, pituitary, and digestive tract; a reduction in pelt weight; or cold injury of the ear. Like the indoor white rats and unlike the outdoor white rats, however, the wild rats showed an hypertrophy of the heart and an increased vascularization of the ear.

The only organ that was not affected by cold in any of the three environmental conditions was the lung.

Judging from the few morphological changes that were found during the winter in wild rats, it appears that these animals were hardly affected by the cold weather. This means either that the wild rats were more resistant to cold than the white rats or that the winter conditions were less severe for wild rats than the cold conditions imposed on white rats when kept individually in a

cold chamber or even when kept in groups outdoors. Most probably both factors were involved. First of all it has been shown that wild rats are much more resistant than white rats to stressful stimuli such as sudden exposure to cold or to high-frequency sound (9, 10, 11). Secondly it could be expected that for rats living free in burrows on a dump where food is plentiful, the cold stress might be less severe than for white rats exposed to cold in cages.

Under these circumstances, it was questionable whether any physiological adjustments took place in wild rats leading to increased cold resistance during the winter. Tests to be reported subsequently will deal with the physiological modifications of wild rats to seasonal changes.

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MAST CELLS IN THE SKIN OF THE EAR OF THE RAT EXPOSED TO COLD[1]

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With the technical assistance of DONNA WRIGHT

Abstract

Mast cells have been counted in ear sections of white rats exposed to 6° C for different periods of time varying from 0 to 16 weeks. There was a greater concentration of mast cells at the tip of the ear than at the base and at the tip more of these cells were lined up at the periphery under the epidermis than internally close to the cartilage. This difference gradually decreased towards the base, where there were more internal cells than peripheral ones. Upon exposure of the rats to cold, the number of mast cells dropped, the peripheral cells disappearing faster than the internal ones, with this differential being greater at the tip than at the base of the ear. Cold injury, like other types of injury, was accompanied by a disappearance of granules in mast cells; however, unlike other types of injuries studied in animals kept at normal room temperature, when cold injury subsided and fibrillogenesis took place there was no concomitant increase in the number of mast cells. The suggestion is made that under cold conditions an excess of ground substance to be stored in the mast cells is not produced during fibrillogenesis. Upon return of the animals to 30° C, the number of mast cells did not start increasing before the 7th day, and this slow recovery suggests a slow mitotic rate.

Introduction

It is well known that when connective tissue is subjected to trauma—mechanical, chemical, thermal, or bacterial—whereby an "acute watery oedema" is produced (1, 2), mast cells promptly release their metachromatic substance into the tissues. Moreover, many authors agree that in acute inflammation the mast cells temporarily disappear (3, 4, 5). Later on, as the acute inflammation subsides and as spindle-shaped fibroblasts elaborate their fibrils, there occurs a progressive increase in the number of mast cells in the affected area; and as long as this proliferative phase persists the local mast cell population continues to increase (6, 7). This pattern of events found in many different kinds of tissue injury brought Riley (2) to state that "fibroplasia, normal or pathological, is consistently followed by the development of tissue mast cells".

Another type of tissue injury, non-freezing cold injury, has been shown recently to produce a typical inflammatory reaction accompanied by oedema and leucocyte infiltration (8). This type of injury, which develops in the ears of white rats during their first 3 or 4 weeks of continuous exposure at 6° C, has also been shown to subside after 4 weeks even when the animals remain in the cold room for another 3 months (8).

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After LeBlanc reported a decrease in the number of mast cells in the ears of rats that had been kept at 6° C for 4 weeks (9), the question arose whether or not the disappearance of acute inflammation mentioned above was accompanied by fibroplasia and reappearance of granules in the mast cells, as had been found for other types of injuries in animals kept at normal temperature. To answer these questions the number of mast cells was counted in tissues from animals used in a previous study (8); in this study the effect of constant low environmental temperature (6° C) on the development and healing of cold injury had been examined.

Experimental Procedures

The animals, and the feeding, housing, and environmental conditions, were given in a preceding paper (8). Ninety-six Sprague–Dawley male rats of an average body weight of 215 g were randomly assigned to 16 groups which were kept at 22° C for 3 days and then transferred to 6° C. Each rat was in an individual cage and had an ad libitum supply of Master Fox Chow and tap water. After 0, 2, 7, 14, 21, 28, 56, 83, and 118 days of exposure to 6° C, groups of five rats chosen at random were killed. A 3×7 mm piece of tissue running radially from the tip to the center of the ear (see Fig. 1A) was cut with scissors from the right ear of each rat. After the usual procedure of fixation in Bouin, dehydration in alcohol, and embedding in paraffin, the tissues were cut longitudinally on the microtome, set at 10 μ, and stained with pinacyanol erythrosinate according to the method described by Bensley (10).

As shown in Fig. 1B, the section was divided into four 1-mm segments and all the mast cells visible in each one of these segments were counted. Since the mast cells close to the epidermis (see No. 1 in Fig. 1C) appeared different, having fewer grains than the deeper ones closer to the cartilage (see No. 2 in Fig. 1C), a differential count was made; all the mast cells within 40 μ of the epidermis were arbitrarily considered as peripheral cells while all the others were called internal cells. With a Bausch and Lomb enlarger, each section was projected on a piece of paper and enlarged 40×. The section was drawn and the area covered by the connective tissue between the cartilage, and the epidermis was measured with a planimeter. By division of the projected image into segments corresponding to 1 mm on the section, the area for each millimeter could then be obtained. Knowing the area, we could calculate the concentration of mast cells in each segment.

Results

At time 0, before exposure of the rats to cold, the total number of mast cells in each segment of ear was greater at the base (4th mm) than at the tip (1st mm) but the concentration was greater at the tip than at the base (Fig. 2).

Upon exposure of the animals to 6° C the number of mast cells gradually decreased in all portions of the ears during the first 3 to 4 weeks, to reach a low level which remained constant for the following 3 months (Fig. 3).

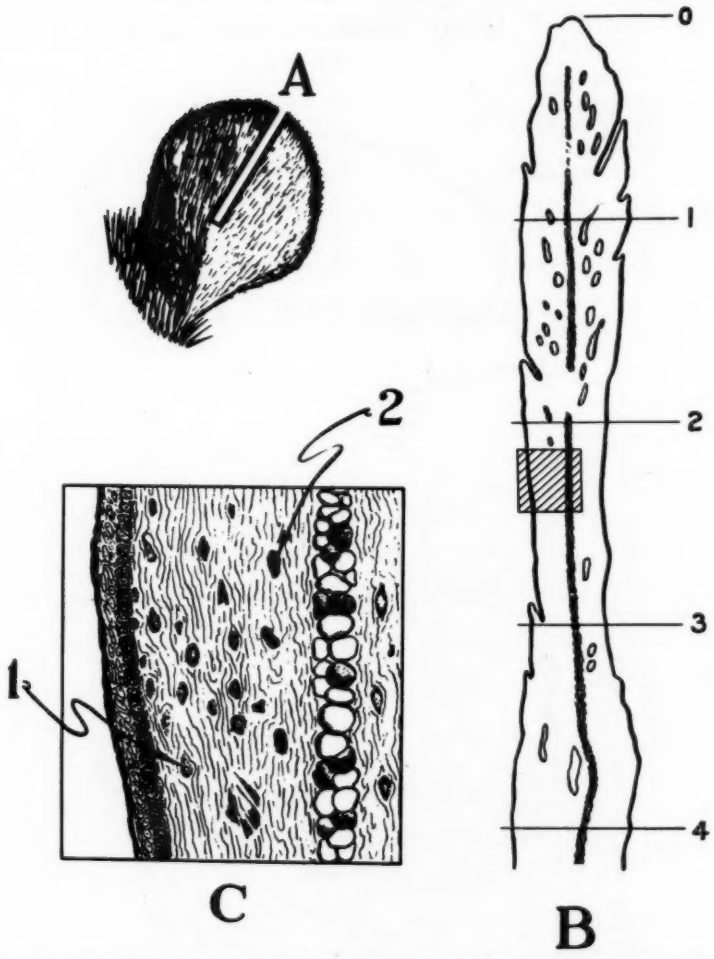

FIG. 1. (A) Location from which the 3×7 mm piece of tissue was taken from the ear; (B) diagram showing the four 1-mm lengths in which cells were counted; (C) 1 = peripheral mast cells within 40 μ of the epidermis; 2 = internal cells.

The rate of mast cell disappearance did not differ significantly from location to location (Table I, first part). In proportion, however, there was a greater drop (60%) in the 1st mm than in the 4th mm (30%).

The average differences for each day between peripheral counts and internal counts were greater in location 1 than in location 2, in 2 than in 3, etc., varying from all positive (peripheral count > internal count) in location 1 to all negative (internal count > peripheral count) in location 4 (Table I, second

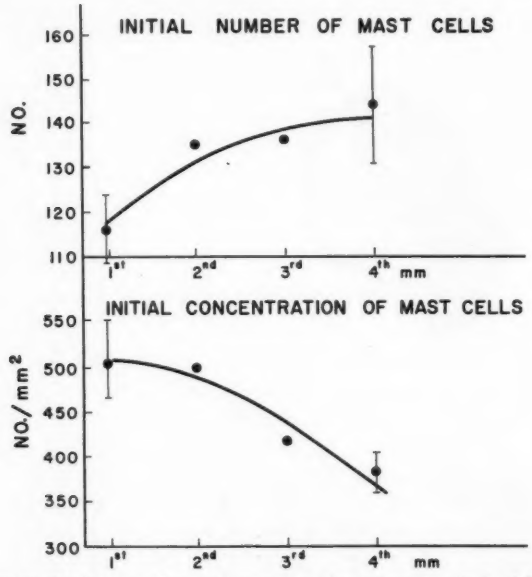

FIG. 2. Initial absolute number and concentration of mast cells in different portions of the ear of rats.

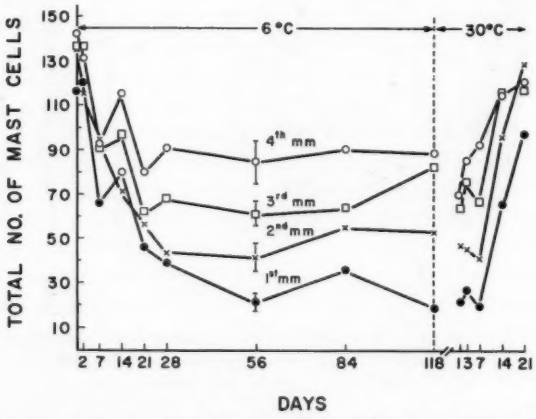

FIG. 3. Changes in the number of mast cells in different portions of the ear in rats kept at 6° C for 118 days and in rats returned to 30° C for 21 days.

part). This part of Table I also shows that the peripheral cells disappeared faster than the internal cells, and that this difference was most pronounced (Fig. 4) in the first 2 millimeters.

Finally, it was found that after 4 months of exposure of the rats to cold, if they were returned to 30° C, the number of mast cells started increasing only

TABLE I

Average mast cell counts

Day	Mast cell count in locations: 1	2	3	4	Day	Mast cell count in locations: 1	2	3	4
	Peripheral+internal					Peripheral−internal			
0	116	135	136	144	0	48	9	0	−10
2	120	116	130	131	2	58	29	6	−11
7	66	95	92	94	7	28	11	−7	−3
14	82	73	94	116	14	33	14	−2	−23
21	47	56	63	81	21	0	−12	−16	−34
28	39	41	67	99	28	5	−7	−5	−14

NOTE: All above averages are based on five animals. Necessary differences between any two averages within the same portion of the table to attain 5% or 1% of statistical significance are 18 and 24 respectively.

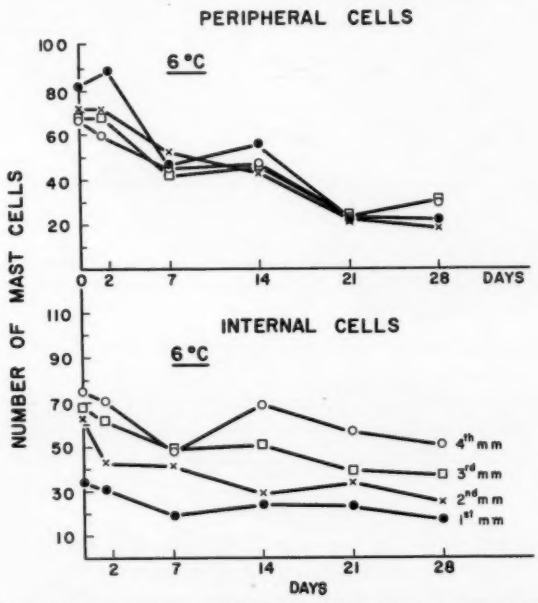

FIG. 4. Changes in the number of peripheral and internal mast cells in different portions of the ear during the first 28 days of exposure at 6° C.

after the 1st week (Figs. 3 and 5). It could also be noticed (Fig. 5) that both peripheral and internal cells reappeared at the same time.

In the last 3 months of cold exposure, while the mast cell population remained constantly low, healing of the cold injury was characterized by an important proliferation of fibroblasts laying down numerous fibrils. This great fibrillogenesis, which could be seen in all cold-acclimated rats, explains the increased ear thickness previously observed in these sections and reported in a first paper on the subject (8). In Fig. 6 are typical pictures, taken at the same magnifica-

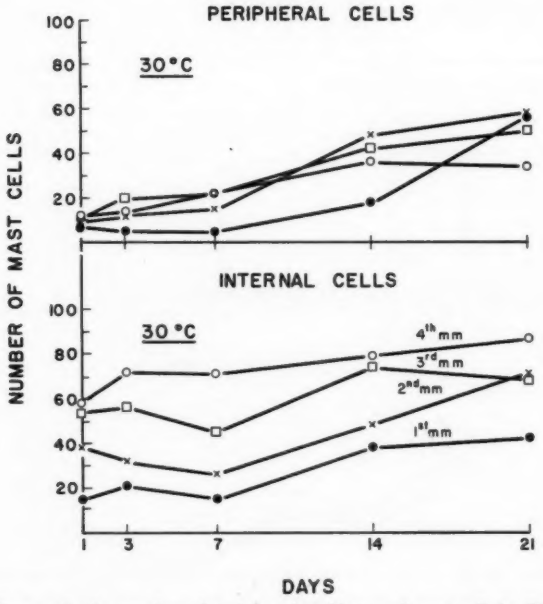

FIG. 5. Changes in the number of peripheral and internal mast cells in different portions of the ear of rats kept at 30° C for 21 days after a previous sojourn of 4 months at 6° C.

tion, of ear sections of warm- and cold-exposed rats. Figure 6A shows a section from the ear of a control rat kept at room temperature and Fig. 6B one from the ear of a rat that had been kept at 6° C for 56 days. In this particular case, the dermis at the tip of the ear was at least four times thicker in the cold-acclimated rat than in the warm-acclimated one.

Discussion

The drop in the number of mast cells during the first 4 weeks of exposure of rats to cold paralleled the gradual blocking of mitotic cells in epidermis and the development of inflammation; both attained their maximum around the same time (2). After the first month, blocking of mitosis ceased and inflammation gradually disappeared and there was no further change in the number of mast cells for the following 3 months.

These results reveal a striking resemblance in the time sequence of both phenomena, that is the drop in the number of mast cells and the development of cold injury. In that respect, cold injury resembles all the other types of injury which have been shown to bring about a drop in mast cell population.

The blocking of mitosis and the disappearance of mast cells both stopped, at least at the tip of the ear, at approximately the time of occurrence of other indices of internal adaptation such as shivering, which completely disappears

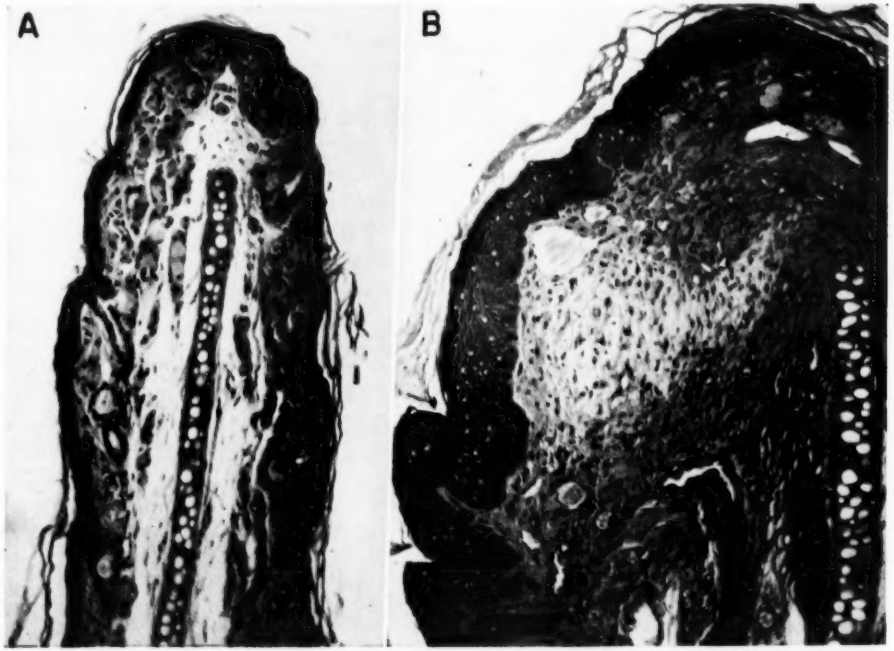

FIG. 6. (A) Section of ear from a control rat. (B) Section of ear from a rat kept at 6° C for 56 days. ×100.

around the fourth week of cold exposure. This also corresponds to the time required for maximal sensitivity to noradrenaline, maximal increase in food consumption, and maximal increase in survival at lethal temperatures. The coincidence in the time at which peripheral adaptation and central adaptation take place is striking but this does not necessarily establish a causal relationship between the two sets of phenomena.

In this study, four observations were made that should be of interest to investigators working specifically on the origin, distribution, and role of mast cells. The first observation is that there were more peripheral cells than internal ones in the 1st mm at the tip of the ear and that this difference gradually decreased in the following locations until it changed sign in the 4th mm at the base of the ear. At that location, there were more internal cells than peripheral ones.

The second observation is that, upon exposure of the animals to cold, the peripheral mast cells disappeared faster than the internal ones at all locations and that this difference in rate of disappearance between the two types of cells varied perceptibly from location to location, being greater at the tip than at the base of the ear. This may mean either that peripheral cells were more labile than internal cells or that under the conditions they were more affected than the internal ones, possibly because the temperature was lower at the periphery than at the center, and lower at the tip than at the base.

The third observation concerns the low and constant level of mast cells during the last 3 months of cold exposure when the signs of cold injury disappeared and fibrillogenesis took place. The present observation reveals that, at least under cold conditions, healing of an inflammatory wound and fibrillogenesis are not necessarily accompanied by an increase in number of mast cells as found by Staemmler (7) and Asboe-Hansen (11) for other types of injury in animals kept at normal temperature. These latter observations even prompted Riley to state in his theory on the role of mast cells that with "the onset of fibrillogenesis, excess ground substance is broken down, rebuilt, and stored in altered, sulphated form (heparin) in the granules of the tissue mast cells, whence it can later be released and be again ingested by cells of the connective tissues" (2). The present results may have two possible explanations: (*a*) the increase in number of mast cells usually found after injury does not really represent a storage of excess ground substance produced during fibrillogenesis but rather a formation of new cells; in cold injury, however, the formation of new cells would be prevented by the cold temperature of the tissue; (*b*) under cold conditions, an excess of ground substance is not produced during fibrillogenesis. The second possibility is the most probable one since other types of cells such as fibroblasts and epidermal cells have been seen to multiply under these cold conditions. It is difficult to understand why duplication of mast cells and not of the others would be completely blocked in cold tissues.

One last observation that merits attention is that upon return of the animals to 30° C the mast cell population started to increase only after the seventh day

and even by the end of the third week the number of mast cells had not returned to what it was before the animals were exposed to 6° C. This retarded recovery indicated that if the mast cells multiply by mitosis, their mitotic rate is very slow indeed.

Acknowledgment

Statistical analysis of the data, provided by the Biometrics Section of this Division, is gratefully acknowledged.

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THE RELATION BETWEEN PHOSPHATE METABOLISM AND THE TRANSPORT OF CATIONS ACROSS THE CELL MEMBRANE IN THE HUMAN ERYTHROCYTE[1]

RHODA BLOSTEIN,[2] DAVID RUBINSTEIN, AND ORVILLE F. DENSTEDT

Abstract

The rate of passage of cations across the red cell membrane, in the direction of the respective ionic gradients, in blood preserved with glucose at 4° C, is not diminished upon the addition of inosine, notwithstanding the induced formation of substantial amounts of phosphate esters, including ATP, in the cells. The movement of cations, however, is retarded on the addition of glucose or inorganic phosphate, and on increase in the concentration of hydrogen ions. It would appear, therefore, that the movement of cations across the membrane, at 4° C, is influenced little, if at all, by the metabolic activity of the cell.

When the temperature of the preserved blood is returned to 37° the rate of movement of cations across the cell membrane against their respective gradients is greatest in cells which had either been preserved or have been incubated with inosine even if the remainder of the inosine has been removed. Inosine is more effective than glucose in bringing about the restoration of cation composition in preserved red cells at 37°. The degree of restoration is greater in the presence of glucose than in its absence. Arsenate inhibits the transport of cations against the gradients. Evidence is given that the capacity of the cells to restore the cation distribution at 37° C is determined largely by the concentration of the phosphate ester intermediates.

Jeanneney *et al.* (1), Maizels and Paterson (2), and many others have shown that when citrated blood is stored in the cold (4–5° C) the erythrocytes undergo a progressive loss of potassium and gain of sodium. The rate of intake of sodium tends to be somewhat greater than that of the loss of potassium, thus giving rise to a progressive net increase in the cation content of the cells during storage. Maizels (3) observed that the addition of glucose to blood retarded the rate of loss of potassium and gain of sodium in the cold. The rate of exchange of these cations can be retarded by increasing the hydrogen-ion concentration of the blood specimen to pH 7 as occurs immediately, for example, when blood is collected into the ACD (acidified citrate–dextrose) medium commonly used in hospital blood banks (4). When the temperature of the cold-preserved specimens is returned to 37° C the rate of metabolism of the cells is correspondingly increased and the sodium is gradually expelled and potassium regained. The ability of the preserved cells to effect this reconstitution at 37° becomes progressively less with prolongation of the period of cold storage. The capacity for reconstitution is best maintained during storage when glucose is present in the preservative medium.

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Contribution from the Department of Biochemistry, McGill University, Montreal, Que. This study represents part of a larger investigation on the preservation of blood supported by the Defence Research Board of Canada Grant No. 9350-01, Project D50-93050-01.

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The ability of erythrocytes to transport cations is believed to be associated with the maintenance of the glycolytic potential of the cells. The addition of inosine to preserved blood induces the synthesis of phosphate ester intermediates in the red cells, thus increasing their metabolic potential (5). It was of interest, therefore, to study the influence of the nucleoside on the movement of the cations across the cell membrane at 4° and at 37°.

Experimental

Composition of Preserved Blood Specimens

Blood from human donors was collected aseptically into the following media for preservation at 4° C:

1. Neutral Citrate Medium (Designated C)

Isotonic (3.2%) solution of trisodium citrate – dihydrate. Proportion of blood to medium, 5:1 (v/v).

2. Acidified Citrate Medium (AC)

Trisodium citrate (0.5 g) and citric acid (0.9 g) in 100 ml of water. Proportion of blood to medium, 5:1.5.

3. Neutral Isotonic Citrate–Dextrose Medium (CD)

Mixture of 1 part of 3.2% trisodium citrate and 0.5 part of isotonic (5.4%) dextrose. Proportion of blood to medium, 5:1.5.

4. Neutral Citrate–Inosine (CI)

Solution containing 3.2% trisodium citrate and inosine to give a final concentration of 2% in the medium. Proportion of blood to medium, 5:1.

5. Neutral Citrate–Dextrose–Inosine (CDI)

Mixture of 1 part of 3.2% trisodium citrate and 0.5 part of a solution containing 5.4% dextrose, and inosine to give a final concentration of 2% in the medium. Proportion of blood to medium, 5:1.5.

In some of the experiments, as indicated later, inorganic phosphate in the form of sodium phosphate buffer (pH 7.5) was added to the blood specimens to give a final phosphate concentration of 5×10^{-3} *M*.

Analytical Procedures

Estimation of Potassium and Sodium

These analyses were done by the "indirect" method with a Baird flame photometer Model DB4, using a lithium chloride solution as the reference standard.

Fractional Analysis of Phosphates

The "labile" nucleotide phosphate (P_L) was estimated according to the method of Lipmann and Crane (6). In addition the following phosphate fractions were determined as described in a former paper (7).

Total phosphate (P_T).—Inorganic phosphate obtained on digestion by sulphuric acid.

Inorganic phosphate (P_I).—The inorganic phosphate normally present in the red cells.

Moderately stable phosphate (P_M).—Represented by the increase in inorganic phosphate on hydrolysis of the red cells for 100 minutes in 1 *N* HCl at 100°, minus the P_L. This fraction is derived mainly from the ester phosphate on the primary alcohol groups of hexose and pentose sugars.

Stable phosphate (P_S).—The ester phosphate, mainly of 2,3-diphosphoglycerate (2,3-DPG) which is not hydrolyzed by treatment with 1 *N* HCl for 100 minutes at 100° C. Thus $P_S = P_T - (P_L + P_M)$.

The inorganic phosphate in each instance was estimated according to the method of Fiske and Subbarow (8).

Other Methods

Glucose (9), ribose (10), and hemoglobin (11) were determined by the methods indicated by the reference numbers.

Incubation Procedure

The following procedure was used in the experiments involving incubation at 37° (see Experiments and Results).

At weekly intervals, the red cells in the cold-preserved blood specimens were uniformly resuspended by gently swirling the contents of the bottles. From each specimen a sample (50 ml) was removed aseptically and the red cells washed 3 times with a 1:1 (v/v) mixture of isotonic NaCl (0.154 *M*) and sodium phosphate buffer (0.11 *M*), pH 7.5, and twice further with isotonic NaCl. A quantity (about 12 ml) of the washed cells from each sample was then added to an ice-cold solution containing 14 ml of isotonic NaCl and 6.0 ml of isotonic (0.154 *M*) KCl. From the uniform cell suspensions three 10.0-ml portions were removed and designated A, B, and C. To these were added phosphate and glycylglycine buffers, $MgCl_2$, glucose, and inosine, as indicated in Table I. A 5-ml portion was removed for analysis and the remainder was incubated at 37° for 4 hours and then analyzed.

TABLE I

Composition of the preparation for incubation of the washed red cells

Sample	Cell suspension (ml)	Sodium phosphate buffer, 0.11 *M*, pH 7.5 (ml)	Glycyl-glycine buffer, 0.11 *M*, pH 7.5 (ml)	$MgCl_2$, 0.1 *M*, (ml)	Glucose added, (mg%)	Inosine added, (mg%)
A	10	0.5	1.0	1.0	—	—
B	10	0.5	1.0	1.0	400	—
C	10	0.5	1.0	1.0	—	500

Experiments and Results

Exchange of Cations between Red Cells and Plasma during Storage at 4°

Blood from one donor was collected into a small quantity of each of the

media C, CD, CI, and CDI to give the proportions indicated in the preceding section on methods. All the specimens were stored at 4°. At intervals during the storage period the cells were uniformly suspended and samples were removed aseptically for analysis. The total content of phosphate esters of the whole blood and the sodium and potassium content of the packed red cells were determined.

A comparison of the rate of efflux of potassium from the cells and the rate of influx of sodium is indicated in Fig. 1.

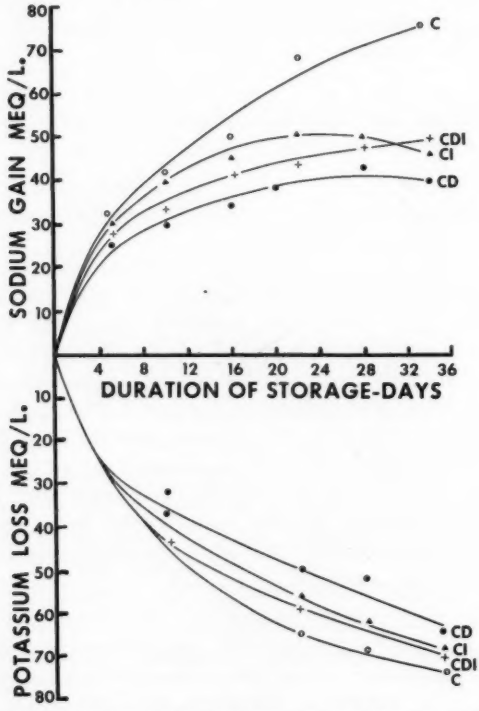

FIG. 1. Change in the sodium and potassium content of human red blood cells during storage in various preservative media at 4° C. C, citrate solution; CD, citrate–dextrose; CI, citrate–inosine; CDI, citrate–dextrose–inosine.

It may be observed that inosine (CI) was less effective than glucose (CD), and glucose along with inosine (CDI) was less effective than glucose alone (CD), in retarding the loss of potassium from the cells during storage. The media with inosine, however, were more effective in maintaining the level of phosphate esters in the cells. The results in Table II for specimens 1 to 4 inclusive show that the concentration of these esters in the cells was increased during storage at 4° in the presence of inosine as reflected in the low concentra-

tion of inorganic phosphate, whereas it was unchanged in the specimens without the nucleoside. The presence of inosine resulted in a rapid esterification of inorganic phosphate, thus reducing the concentration of the latter to a low level during storage. In view of this effect of inosine, it was of interest to determine whether the addition of inorganic phosphate would influence cation transport. The concentration of the added phosphate was as indicated with specimens 5–8 in Table II. Comparison of the concentration of the inorganic phosphate in specimens 7 and 8 with that in specimens 5 and 6 on the 35th day of storage shows that esterification of phosphate was brought about in the presence of inosine.

TABLE II

Influence of the preservative medium on the esterification of inorganic phosphate in blood preserved at 4° C for 35 days

Specimen No.	Preservative medium	Inorganic phosphate (μmoles/ml)		
		Day 0	Day 35	Net change
1	C	0.8	7.3	+6.5
2	CD	0.8	6.7	+5.9
3	CI	0.6	1.0	+0.4
4	CDI	0.4	0.9	+0.5
5	C+P_I	6.2	13.0	+6.8
6	CD+P_I	6.4	12.5	+6.1
7	CI+P_I	6.1	3.1	−3.0
8	CDI+P_I	7.2	3.6	−3.6

The influence of the added inorganic phosphate on the rate of exchange of sodium and potassium is evident from a comparison of the results with the added phosphate, indicated in Fig. 2, with those in Fig. 1. The data in both figures were obtained on the same blood donation. It is evident that the order of the effectiveness of the various preservative media in retarding the loss of potassium during storage was not altered by the addition of inorganic phosphate despite the higher level of phosphate esters in inosine-containing specimens. However, if comparison be made of the magnitude of the change in cation concentration on a given day (e.g. the 16th day) of storage, as indicated in Table III, it may be observed that the addition of the inorganic phosphate had retarded the rate of loss of potassium and gain of sodium. It is evident that in

TABLE III

Influence of added inorganic phosphate on the sodium and potassium changes in blood preserved at 4° C for 16 days

Preservative medium	Potassium loss (meq/l.)		Sodium gain (meq/l.)	
	Control	+Phosphate	Control	+Phosphate
C	44.6	39.0	49.5	46.7
CD	35.3	31.8	34.5	29.5
CI	45.2	44.0	44.0	42.1
CDI	47.8	45.2	41.5	38.0

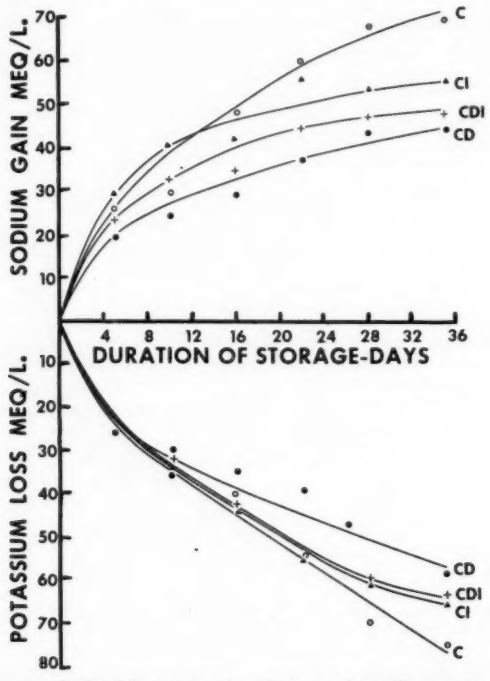

FIG. 2. Influence of added inorganic phosphate on the sodium and potassium content of red blood cells during storage at 4°. C, control (citrate solution); CP, citrate with added phosphate; ACD, control (acidified citrate–dextrose); ACDP, ACD with added phosphate.

the case of the specimens without inosine, the presence of added phosphate decreased the rate of passive loss of potassium and gain of sodium. The effect of added inorganic phosphate was less marked in the specimens containing inosine.

In view of the apparent absence of cellular metabolic control of ion transport at 4° as indicated by the lack of correlation between the change in the concentration of phosphate esters and the transport of cations against the ionic gradient, it was of interest to see whether the addition of a metabolic inhibitor would influence the movement of cations.

Blood was collected into isotonic citrate solution and the specimen divided into two equal portions. One served as the control while, to the other, fluoride was added to give a final concentration of 5×10^{-3} *M*. The results in Table IV show that, notwithstanding the complete inhibition of glucose utilization by the fluoride, the rate of loss of potassium was retarded.

A similar retardation of the rate of cation exchange was evident when the metabolic activity of the red cells was depressed by an increase in the concentration of hydrogen ions such as occurs when blood is collected into the ACD medium commonly used for blood preservation. The curves in Fig. 3 give a

TABLE IV

Influence of fluoride on the concentration of potassium and glucose in red cells during storage of blood in isotonic citrate solution at 4° C

Storage period (days)	Cell potassium (meq/l.)		Blood glucose (mg%)	
	Control	With added NaF*	Control	With added NaF*
0	95.7	97.1	78	77
2	78.6	87.5	51	76
5	66.1	78.0	22	71
9	59.4	68.0	1	73

NOTE: The data in the above table are taken from the Ph.D. thesis of S. Fishman, Department of Biochemistry, McGill University, 1953.
*Final concentration of NaF, 0.005 *M*.

comparison of the rate of cation exchange between the red cells and plasma in specimens preserved in the acidified citrate (AC) and in neutral citrate (C) media during storage at 4° C.

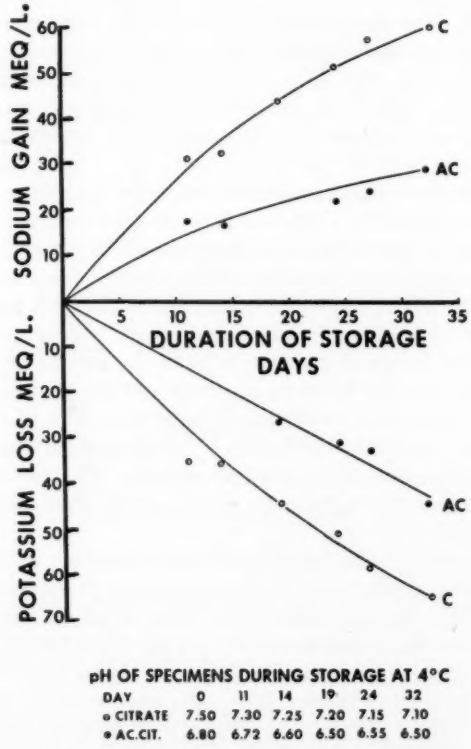

FIG. 3. Influence of hydrogen-ion concentration of the preservative medium on the movement of cations across the red cell membrane in blood during storage at 4°. C, control (isotonic citrate solution); AC, acidified citrate.

It is evident that there was a marked decrease in the rate of passive diffusion of cations in the acidified sample (ACD).

Influence of the Substrate on the Capacity of Cold-preserved Red Cells to Restore the Cation Composition towards the Normal when Incubated at 37°

Blood was preserved in isotonic citrate solution for 36 days at 4° C. At intervals during the period, samples were withdrawn, the red cells washed (see Incubation Procedure), and divided into three equal portions, A, B, and C. Sample A, without the addition of substrate, served as the 'control'. To B glucose was added, and to C, inosine (Table I). Sterile precautions were taken throughout the manipulations. The samples then were incubated at 37° for 4 hours. Portions were removed before and after the incubation and analyzed for sodium, potassium, glucose, ribose, and inorganic phosphate.

The curves in Fig. 4 indicate the behavior of sodium and potassium in the specimens. It is evident that only until the 5th day of storage were the cells in the control sample able to effect a significant degree of cation redistribution during incubation. The cells in sample B with added glucose, on incubation, were able to eliminate sodium and take up potassium until the 12th day of storage. The cells in sample C (containing inosine), throughout the entire period of cold storage (60 days), maintained the capacity to expel sodium and regain potassium on incubation at 37°. Additional analytical data for these samples are given in Table V.

The capacity of the cold-preserved erythrocytes to utilize glucose on incubation at 37° is indicated in Table V. It is evident that the capacity fell off sharply in the samples taken between the 11th and 18th days. The capacity of the cells in the same specimen to utilize ribose (derived from the added inosine) at 37°, on the other hand, reached a maximum at the 18th day and remained strong throughout the entire period of storage. It is clear, furthermore, that the concentration of inorganic phosphate in all the samples A and B until the 11th day of storage was increased on incubation of the samples at 37°. Thereafter, the concentration decreased slightly (as indicated by the negative values in the table). The cells from the samples C (incubated with inosine) effected a pronounced reesterification of inorganic phosphate. This removal of free phosphate reached the greatest magnitude in the sample that showed the highest utilization of ribose.

In view of the relationship between the concentration of phosphate esters and cation transport another study was undertaken to ascertain the influence of the level of ATP in the red cells on the behavior of cations. A specimen of blood in CD medium was preserved at 4°. By the 26th day of storage the concentration of the phosphate ester intermediates (including ATP) in the cells had fallen to a low concentration. The cells were washed and divided into three samples A, B, and C, containing respectively, no substrate, added glucose, and added inosine. The samples were incubated and analyzed as in the previous experiments.

TABLE V

Metabolic changes in cold-preserved specimens of red cells on incubation at 37° C

Storage period (days)	Glucose utilization (μmoles per mg of Hb)	Ribose utilization (μmoles per mg of Hb)	Net changes in inorganic phosphate (μmoles per ml of blood)		
	Sample B	Sample C	Sample A	Sample B	Sample C
0	24.4	74.2	+25.4	+ 7.2	−30.9
4	19.6	96.0	+33.6	+22.8	−19.8
7	16.6	47.7	+26.4	+19.5	−20.8
11	25.2	61.3	+10.2	+22.0	−35.7
18	5.6	102.2	− 2.3	− 3.0	−59.5
26	9.1	85.1	+ 1.4	− 3.5	−57.0
36	5.3	97.5	− 4.0	− 4.0	−45.5

NOTE: Preserved specimen: Blood collected into a mixture of isotonic trisodium citrate (3.2%) in the volume: volume proportion 5:1, and specimen stored at 4° C. Composition of samples A, B, and C as indicated in Table I (see Methods).

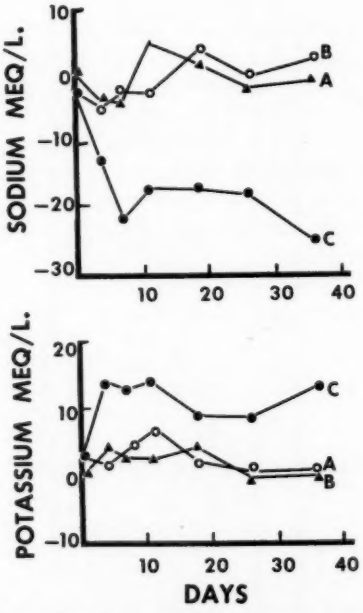

FIG. 4. Net change in sodium and potassium content of red cells from citrated cold-preserved blood, on incubation at 37° (with added substrates). (A) Control (isotonic citrate, no added substrate); (B) with added glucose, final concentration 400 mg%; (C) with added inosine, final concentration 500 mg%.

It is evident from the data in Table VI that the cells in specimen A were unable to effect any resynthesis of phosphate intermediates or movement of cations on incubation at 37°. To the contrary, a further hydrolysis of the

TABLE VI

Cation movement and the generation of high-energy phosphate compounds in 26-day-old cold-preserved red cells on incubation at 37° C in the presence of inosine

Incubation period (hours)	Sodium (meq/l. cells)			Potassium (meq/l. cells)			Labile phosphate (μmoles/ml cells)		
	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
0	36.5	36.8	37.6	54.0	53.0	54.5	0.79	0.79	0.79
4	36.5	32.2	24.8	54.0	52.6	59.4	0.57	0.78	2.25
Net change during incubation	0.0	−4.6	−12.8	0.0	−0.4	+4.9	−0.22	−0.01	+1.46

NOTE: Blood stored at 4° C in CD medium. Sample A without added substrate. Sample B with added glucose (500 mg/100 ml). Sample C with added inosine (500 mg/100 ml).

residual ATP ('labile' phosphate) occurred during the incubation. The cells in specimen B, with added glucose, effected an expulsion of sodium of small magnitude but no uptake of potassium. No change occurred in the ATP concentration during incubation, that is, the rate of breakdown of ATP presumably was equal to the rate of phosphorylation of ADP. In marked contrast to the behavior of specimens A and B, the cells in specimen C, on the addition of inosine, underwent a threefold increase in the reserve of ATP and effected a marked expulsion of sodium and uptake of potassium.

In view of the findings in the preceding study it was important to determine whether the replenishment of phosphate esters induced by inosine at 4°, but which had no effect on cation movement at the low temperature, could confer upon the preserved cells a potential for reconstituting the ionic composition when the temperature is restored to 37° even if inosine be removed. This would be comparable to the conditions prevailing if the preserved cells were placed in the circulation.

Blood was collected in CD and CDI media and stored at 4°. Periodically samples were removed and prepared for incubation as previously described. The cells, after having been washed to remove the inosine, gave a negative orcinol test for the presence of pentose. They were then incubated *without* the addition of substrate at 37° for 4 hours. Analysis for the sodium and potassium content of the cells was performed at the beginning and at the end of the incubation. The results are indicated in Fig. 5. It is evident that the capacity of the cold-preserved red cells to effect a redistribution of sodium and potassium

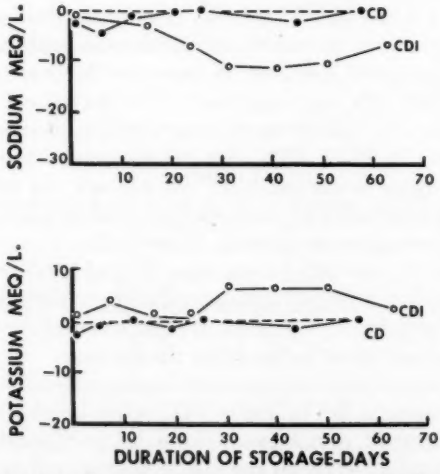

FIG. 5. Net change in sodium and potassium content of red cells from cold-preserved blood, on incubation at 37° without added substrate. CD, blood preserved in citrate–dextrose at 4° up to 60 days. CDI, blood preserved in citrate–dextrose–inosine.

on incubation, in the case of the CD specimen, fell to zero by the 20th day of storage. In the CDI-preserved specimen, on the contrary, the cells retained the capacity in high degree to the end of the storage period (64th day). The preservation of the capacity to redistribute cations at 37°, therefore, is related to the maintenance of levels of phosphate esters under the influence of inosine, and not to any direct action of inosine on cation movement.

The results obtained in the preceding experiment indicated that the formation of phosphate esters during preservation of erythrocytes with inosine conferred upon the cells the ability to expel sodium and take up potassium against the respective concentration gradients at 37° even though the inosine had been removed. It was of interest, therefore, to ascertain whether this capacity would be present also in preserved cells depleted of potassium but with a relatively high content of phosphate esters.

The experiment was carried out in the following manner:

Blood was preserved in CD medium for 21 days at 4° to permit the concentration of potassium and phosphate esters to fall to a low level. The cells were washed 4 times with a mixture (v:v/1:1) of isotonic sodium chloride (0.154 *M*) and sodium phosphate buffer (0.11 *M*) at pH 7.5. After the final centrifugation the packed cells were divided into two equal portions (35 ml) designated A and B. Both lots were suspended in a potassium-free medium, as indicated in the first two columns in Table VII. Sample A served as the control while, to B, inosine was added to induce synthesis of phosphate esters. The purpose in using the potassium-free medium was to minimize the loss of sodium during the subsequent incubation treatment, since it is known that red cells have little ability to expel sodium in the absence of potassium (12). After incubation of the specimens for 2 hours at 37° the cells in both samples were again washed 4 times as described above to remove the incubation medium and particularly to remove the inosine from specimen B (tests for the presence of pentose in a portion of the packed cells were negative). The packed cells in both samples were resuspended in a potassium-containing medium, as indicated in the third and fourth columns in Table VII, and the specimens designated A and B, respectively, were again incubated at 37° for 3 hours. At intervals during the period, samples were removed for analysis of the red cells for content of sodium and potassium. The results are given in Table VIII.

It is evident that the red cells in specimen B (which had received the initial incubation with inosine) acquired a strong capacity to expel sodium (7.7 meq/l. of cells) and regain potassium (5.5 meq/l. of cells) whereas the cells in the control specimen A_1 had no ability to redistribute the cations.

A further experiment was performed with a cell preparation analogous to that designated "Experimental B" in Table VII. After incubation for 2 hours at 37°, the cells were washed, divided into two equal portions designated B_{1a} and B_{1b}, and suspended respectively in the media represented in specimens A and B, in Table VII. To B_{1a}, arsenate was added to block the formation of ATP at the level of triosephosphate dehydrogenase. The specimens were incubated

TABLE VII

Composition of preparations for incubation

Ingredients of mixture	Potassium-free media for initial incubation (ml)		Potassium-containing media for reincubation (ml)	
	Control A	Experimental B*	Control A_1	Experimental B_1
Washed, packed red cells	35.0	35.0	32.0	32.0
NaCl (0.154 *M*)	6.0	6.0	33.0	33.0
Sodium phosphate buffer (0.11 *M*), pH 7.5	6.0	6.0	4.5	4.5
KCl (0.154 *M*)	0.0	0.0	18.0	18.0
Glycylglycine buffer (0.11 *M*), pH 7.5	2.0	2.0	12.0	12.0
$MgCl_2$ (0.1 *M*)	1.5	1.5	4.5	4.5
Inosine (mg)	0.0	250	0.0	0.0

*After the initial incubation the cells were washed twice with 4 volumes of the NaCl – phosphate buffer mixture (v:v = 1:1) and then twice with 4 volumes isotonic NaCl.

TABLE VIII

Influence of incubation of cold-preserved red cells in a potassium-free medium containing inosine, on the capacity of the cells to redistribute potassium and sodium on reincubation in a potassium-containing medium

Sample	Initial incubation	Reincubation period (hours)	Sodium (meq/l. of cells)	Potassium (meq/l. of cells)
A_1	Without inosine	0	40.5	56.5
		1.5	39.8	57.8
		3	40.0	56.8
		Net change	−0.5	+0.3
B_1	With added inosine (500 mg%)	0	39.4	56.5
		1.5	35.1	61.5
		3	31.7	62.0
		Net change	−7.7	+5.5

TABLE IX

Influence of arsenate on the behavior of sodium, potassium, and phosphate fractions in red cells with an initially high content of ATP, ADP, and sodium

Sample	Period of incubation (hours)	Sodium (meq/l. cells)	Potassium (meq/l. cells)	P_I (μmoles P_I/ml blood)	P_S	P_L
B_{1a} (with arsenate)*	0	35.5	79.5	73.6	26.7	11.6
	3	35.3	80.1	115.7	28.2	8.4
		−0.2	+0.6	+42.1	+1.5	−3.2
B_{1b} (without arsenate)	0	35.5	78.0	73.6	26.7	11.6
	3	32.7	82.4	102.0	25.1	11.8
		−2.8	+4.4	+28.4	−1.6	+0.2

NOTE: The original specimen of blood had been preserved in ACD medium such that the ratio (v:v) of blood: acidified citrate:glucose was 5:1:1.
*Final concentration of arsenate 3×10^{-3} *M*.

for 3 hours at 37° and at intervals the cation content of the cells was determined. The analytical results are given in Table IX.

It is clear from a comparison of behavior of the specimens that the decrease in the concentration of ATP in the presence of arsenate was accompanied by a pronounced decrease in the capacity of the cells to expel sodium and regain potassium at 37°.

Discussion

Cation Movement at 4° C

Our study has shown that an increase in the metabolic potential of preserved erythrocytes at 4° under the influence of purine nucleoside, as represented by the induced synthesis of organic phosphate intermediates and ATP, has little or no influence on the movement of potassium into, or sodium out of, the cells at that temperature. Furthermore, if metabolic control over movement of the cations were operative in the cold, one would expect to find an increase in the rate of escape of potassium and entry of sodium upon inhibition of glycolytic activity. When glycolysis was inhibited with 0.005 *M* fluoride, this did not occur; on the contrary, the rate of loss of potassium was retarded. It may be that fluoride directly or indirectly influences the properties of the cell membrane.

There is evidence from our study that the behavior of potassium and sodium in the red cell at 4° is influenced much more strongly by the concentration of inorganic phosphate in the blood than by the level of the phosphate esters. Thus an increase in the concentration of inorganic phosphate, such as may be produced by addition of phosphate to the external medium, tended to retard the rate of loss of potassium and uptake of sodium. Lowering of the concentration of inorganic phosphate, as may be induced under the influence of inosine by esterification of the phosphate, tends to increase the rate of *passive* cation exchange in the cold. This influence of inosine is evident from a comparison of the results in the presence of inosine with those in the absence of the nucleoside in Figs. 1 and 2 and in Table II.

An apparent contradiction of the foregoing observation occurred in the behavior of the specimen in which inosine was present with citrate as compared to that with citrate alone. For example, although the data in Table II indicate that in the presence of inosine there was only a slight increase in the inorganic phosphate compared to that in the sample containing citrate alone, nevertheless the results in Figs. 1 and 2 show that the efflux of sodium was retarded in the specimen containing inosine (CI). We are inclined to attribute the difference in behavior to the slightly higher hydrogen-ion concentration (production of lactic acid) in the presence of inosine. Our findings confirm the observation of Rapoport (4), in 1947, that the rate of loss of potassium is retarded when red cells are preserved in acidified citrate dextrose (ACD) medium compared to that with the C or CD medium. Rapoport suggested that the superior retention of potassium by the cells may be related to the superior maintenance of ATP

with the ACD medium. The findings reported here, however, show that the level of ATP in the cells, in itself, is not the factor responsible for the maintenance of cation gradients. It seems probable that the properties of the membrane also are influenced by the hydrogen-ion concentration and other factors.

Cation Movement in Cold-preserved Erythrocytes on Restoration of the Temperature to 37°

When the temperature of cold-preserved blood specimens is returned to 37° a movement of sodium and potassium occurs across the red cell membrane against the respective ion gradients. The rate and extent of this active ion transport appears to be directly related to the increase in the metabolic activity of the cells. Thus inosine, which, in contrast to glucose, can readily be metabolized, induced a pronounced esterification of inorganic phosphate, uptake of potassium, and expulsion of sodium. Red cells, stored as long as 90 days at 4°, retain the capacity to utilize inosine (13). Harris and Prankerd (14), with human red cells, and Kirschner and Harding (15), with hog cells, showed that the adenosine likewise influences cation transport across the cell membrane. Adenosine readily undergoes deamination in the cells before it undergoes phosphorolysis (13).

Inosine, thus formed, or when added to blood, yields ribose phosphate without the utilization of ATP. That the influence of inosine in restoring the distribution of cation toward the normal in preserved red cells is related to the concentration of phosphate ester intermediates, was shown by inducing the formation of these esters in preserved cells through the action of inosine, and then removing the remaining inosine. As indicated in Fig. 5, the red cells on further incubation at 37° were able to accumulate potassium and expel sodium even without the addition of substrate.

Whittam (16), several years ago, observed the correlation between the rate of decrease in the concentration of ATP and the diminution in the rate of uptake of potassium in red cells. Similarly, Post *et al.* (17) noted a correlation between the influence of various factors on ATPase activity and cation transport. The dependence of transport upon the metabolic activity of the cell was shown further in our study by using arsenate to accelerate the breakdown of phosphate intermediates and to inhibit the formation of ATP. Concomitantly with the fall in the concentration of ATP, the capacity of the cells for cation transport was decreased. Eckel (18) and Fishman (19) have shown that inhibition of glycolysis with fluoride in concentration of the order of 0.005 M increases the rate of passive diffusion of potassium from the red cell at 37°.

The findings in our study show that whereas the transport of cations across the red cell membrane against an ionic gradient at 37° is an active process controlled by the metabolic activity of the cell, the movement at 4° is a passive process and occurs with the gradient. The uptake of potassium, whether it be dominant over, or secondary to, the expulsion of sodium, is an active process under metabolic control. The Q_{10} of potassium transport is about 2.5 whereas

that of passive diffusion with the concentration gradient approaches unity. Thus, as the temperature of the blood specimen is lowered, the rate of active transport is decreased more rapidly than that of the passive diffusion. At 4° active transport virtually ceases.

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EFFECTS OF ALIPHATIC ALCOHOLS AND FATTY ACIDS ON THE METABOLISM OF ACETATE BY RAT LIVER SLICES[1]

E. MAJCHROWICZ AND J. H. QUASTEL

Abstract

The effects of the addition of normal aliphatic alcohols and fatty acids on the metabolism of acetate by rat liver slices have been investigated, with particular reference to the formation, from acetate-1-C^{14}, of $C^{14}O_2$ and radioactive lipids, proteins, and fatty acids. Whereas the addition of unlabelled acetate causes a fall in the rate of formation of $C^{14}O_2$, the decrease, at low concentrations, is less than that calculated for isotopic dilution. This is probably due to the fact that with an increased concentration of acetate there is an increased rate of acetate oxidation. However, the addition of ethanol causes a larger fall in the rate of formation of $C^{14}O_2$ than would be expected if the alcohol were converted to acetate. This points to some inhibition by ethanol of acetate oxidation, a conclusion borne out by the inhibitory effects of *n*-alcohols on total CO_2 formation (in the absence and in the presence of added acetate) by rat liver slices. The fact, however, that the inhibitory effect of ethanol reaches a maximum at 5 mmolar and is constant to 50 mmolar points against a major inhibition due to ethanol per se. The results would be consistent with the conclusion that ethanol, *at low concentrations*, is more quickly converted into acetyl-CoA than acetate itself and that its speed of conversion into acetyl-CoA reaches a maximum at about 5 mmolar with rat liver slices. *n*-Propanol is much more inhibitory than ethanol but its effect is quantitatively identical with that of an equivalent quantity of propionate. Propionate and *n*-propanol are presumably rapidly converted to propionyl-CoA (or methyl malonyl-CoA), which is the effective inhibitor of acetate oxidation by its competition with acetyl-CoA. Propanol also shows direct inhibitory effects on acetate oxidation in rat liver slices. *n*-Butanol, *n*-pentanol, and *n*-hexanol have inhibitory effects identical with those of equivalent concentrations of corresponding fatty acids. These facts point to the oxidation, in rat liver, of aliphatic alcohols and fatty acids to propionyl-CoA or acetyl-CoA which inhibit $C^{14}O_2$ formation from acetate-1-C^{14} by competition or isotopic dilution. Alternations of inhibition occur between the odd- and even-numbered carbon alcohols and fatty acids which may be explained by the formation of mixtures of acetyl-CoA and the highly inhibitory propionyl-CoA from the long-chain alcohols and acids. Tribromoethanol is less inhibitory than propanol at equivalent concentrations though it is an effective inhibitor of acetate oxidation. Allyl alcohol is a much more potent inhibitor. Ethanol inhibits incorporation of radioactivity of acetate-1-C^{14} into fatty acids in rat liver, the inhibition being approximately equal to, or possibly less than, that due to an equivalent quantity of acetate. This may be explained on the basis of isotopic dilution, by acetyl-CoA derived from the alcohol, with radioactive acetyl-CoA undergoing synthesis to radioactive fatty acids. Thus ethanol, at the concentrations investigated, by taking part in fatty acid synthesis, inhibits acetate conversion to fatty acids. Glucose shows the largest effect in stimulating fatty acid synthesis in the liver from acetate, fructose is less effective, and sorbitol shows no effect. The aliphatic alcohols inhibit incorporation of radioactivity from acetate-1-C^{14} into liver lipids and proteins, propanol and pentanol showing the largest effects. There is an alternation between odd- and even-numbered aliphatic chains. The disappearance, or utilization, of acetate by rat liver slices is inhibited by the aliphatic alcohols, alternations between the odd- and even-numbered chains again being observable. The results are explained as being partly due to isotopic dilution and competition by acetyl-CoA and propionyl-CoA, and partly due to suppressions of acetate metabolism by the alcohols.

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Introduction

Recent investigations (1, 2) have shown that the addition of ethanol at small concentrations diminishes the rate of oxygen consumption of rat brain cortex slices respiring in a glucose phosphate medium when this has been stimulated by the presence of 100 meq/l. potassium ions. Potassium-stimulated respiration of rat brain cortex slices is much more affected by ethanol at low concentrations than the normal respiration, the concentrations being of the same order as those required to bring about the narcotic state in the rat. In fact, unstimulated rat brain respiration may be increased by the presence of ethanol (1). These results have been confirmed by Fischer (3), Sutherland *et al.* (4), and Wallgren and Kulonen (5), who have shown that ethanol causes a decrease in the respiration of electrically stimulated brain tissue in presence of glucose.

The inhibitory effects of alcohols increase markedly as the length of the carbon chain increases and with increase of their concentration, and the potassium ion stimulation of brain cortex respiration is diminished or abolished by concentrations of alcohols that have little effect on the unstimulated respiration (2). *n*-Pentanol is much more effective than ethanol in effecting an inhibition of the stimulated respiration and there seems to take place a rapid establishment of equilibria between the alcohols and the components that influence the brain respiratory system.

A study has been made of the effects of aliphatic *n*-alcohols on the oxidation and the utilization of acetate in normal rat liver slices. The results of this work are reported in this paper.*

When ethanol is oxidized in the liver cell it yields eventually acetyl-CoA, which contributes to the pool of acetyl-CoA derived from fats, carbohydrates, and amino acids. Similarly, other aliphatic alcohols yield fatty acids or their acyl-CoA derivatives that ultimately form propionyl-CoA and acetyl-CoA, which merge with the pool of acetyl-CoA or which exercise true inhibitions (6, 7, 8, 9). The effects, therefore, of aliphatic fatty acids on the oxidation of acetate-1-C^{14} to $C^{14}O_2$ and on the total CO_2 formation have been studied and compared with those of the corresponding alcohols.

Materials and Methods

Wistar male albino rats, weighing between 100 and 200 g and fed ad libitum up to the time of the experiment, were killed by a blow on the head and were then decapitated and exsanguinated. The livers were rapidly removed and submerged in an ice–saline mixture. Slices (not more than 0.5 mm thick) were cut using a chilled Stadie-Riggs knife microtome, surface slices being discarded. Slices to be used were kept on a covered ice-cooled Petri dish, then quickly weighed on a torsion balance, and transferred into prepared Warburg mano-

*Some of these results, and the methods employed, are described in a thesis submitted by one of us for Ph.D. (McGill University) (22). A brief account of them was given at the 26th International Congress on Alcohol and Alcoholism at Stockholm, August 1–5, 1960.

meter flasks containing ice-cold medium (total volume 3 ml). Usually two slices were used, weighing a total of 120–180 mg wet weight.

Incubations were carried out in a physiological medium with 10 mmolar sodium phosphate buffer at pH 7.4, using standard Warburg manometric technique (14). The final concentrations of salts in the medium were NaCl, 141 mmolar; KCl, 5.7 mmolar; $CaCl_2$, 3 mmolar; KH_2PO_4, 1.4 mmolar; $MgSO_4$, 1.4 mmolar. This medium was used, without further additions, for measurements of metabolism with and without the various substrates indicated in the experimental tables. The center wells of the flasks contained 0.15 ml of 20% KOH on a roll of filter paper for absorption of CO_2. The vessels after being attached to the manometers were gassed at a slow rate with pure oxygen for 3 minutes. After 1 hour incubation period at 37° C, trichloracetic acid (0.2 ml, 30%) was tipped from the side arm into the main compartment to stop the reaction and the vessels were shaken for another 30 minutes in order to expel any carbon dioxide absorbed in the incubation medium.

Assay of Radioactive Carbon Dioxide

The contents of the center wells of the Warburg vessels were transferred carefully with the addition of water into centrifuge tubes which were fitted with rubber stoppers and left overnight. The filter paper was then removed and washed and two drops of 2 *M* NH_4Cl and 0.5 ml of saturated $BaCl_2$ solution were added. After 1 hour, the precipitated $BaCO_3$ was washed twice with 10 ml water and once with 10 ml acetone. Finally the $BaCO_3$ precipitate was resuspended in 0.3 ml of acetone and transferred into a weighed aluminum planchette. The centrifuge tube was washed with another four portions of acetone and the washings were added to the planchette. The planchettes were dried on a hot plate, weighed, and the radioactivity was assayed by counting in a gas flow Tracerlab counter. Since the total weight of $BaCO_3$ seldom exceeded 5 mg per plate no correction was made for self-absorption.

Measurement of Total Carbon Dioxide

The amount of total respiratory CO_2 was found by conversion of the weight of $BaCO_3$ used for $C^{14}O_2$ assay into free CO_2 (allowing for the blank on the KOH used).

Assay of Radioactivities of the Proteins and Lipids

The procedure adopted was essentially that of Siekievitz (10) with the modification of Lindan *et al.* (11). After acidification and liberation of CO_2 from the contents of the manometer flask, the tissue slices and the medium were mixed with an equal volume of water and homogenized with a Teflon pestle. Four milliliters of 30% trichloracetic acid was then addded, and the suspension rehomogenized and centrifuged. The deposit was washed twice with 10 ml of 6% trichloracetic acid. Subsequent extractions were carried out with 4 ml of 95% ethanol, 4 ml ethanol–ether mixture (3:1, v/v) at 60° C for 10 minutes, and 4 ml ether. The last three extractions brought into solution the lipids and alcohol-soluble proteins. These extracts were made up to a volume

of 7 ml, 1-ml aliquots were transferred to planchettes, and 2 drops of 0.1 *N* NaOH were added to prevent the loss of volatile fatty acids; they were then dried, and the radioactivities of the lipids and alcohol-soluble proteins were measured.

The insoluble material left from this treatment was extracted twice with 10 ml 6% trichloracetic acid. The first extraction was carried out at 90° C for 10 minutes and the next one at room temperature. Finally, the proteins were resuspended in 95% ethanol and either 0.5-ml or 1.0-ml aliquots were transferred on weighed aluminum planchettes, dried, and weighed, and their radioactivities were measured. It was found that plating of the protein in 95% ethanol resulted in even distribution and firm adhering of the particles to the aluminum planchette.

Purification of Alcohols

The alcohols were redistilled from an all-glass apparatus, which was cleaned in chromic acid. The fractions of the distillate used for the experiments were collected after a relatively large portion of the alcohol had been distilled. Furthermore, the purity of all distilled alcohols and the purity of the solutions were checked by employing a specially devised modification of the gas–liquid chromatographic technique.

Assay of Utilization of Acetate-1-C^{14}

The radioactivity of the incubation medium at the start of each experiment was measured by plating 0.1 ml of the solution with 0.1 ml sodium hydroxide, counting, and then correcting for the total volume of the medium (3 ml).

For the measurement of the radioactivity of the medium at the end of the incubation, the vessels were detached from the manometers, placed in an ice mixture, and thoroughly cooled. The medium was transferred to centrifuge tubes and spun with cooling of the centrifuge cup until a clear supernatant was obtained. Two sets of stainless steel plates were prepared containing 0.1 ml of the supernatant. To one set 0.1 ml of 0.1 *N* sodium hydroxide was added, and to the second set 0.1 ml of 0.1 *N* hydrochloric acid was added. The plates were dried and their radioactivities were measured. The difference between the activities of the two results represents the radioactivity of free acetate. The total radioactivity disappeared from the medium was determined by subtracting the radioactivity of the free acetate left in the medium after incubation from the radioactivity at the start of the experiment. The utilization of radioactivity is given in Table XI in terms of counts per minute per milligram dry weight of tissue.

Experiments were carried out, in a routine manner, in duplicate and repeated several times. Most of the following tables give typical results, but in some the mean deviations from the average are given. The mean deviation rarely exceeded $\pm 10\%$.

Results

Effects of Ethanol on Conversion of Acetate-1-C^{14} into $C^{14}O_2$

The typical results in Table I show the suppressing effect of ethanol on acetate-1-C^{14} conversion to $C^{14}O_2$ by rat liver slices during a 1-hour incubation period in phosphate medium, pH 7.4. With increase of ethanol concentration

TABLE I

Effects of ethanol and sodium acetate on the formation of $C^{14}O_2$ by rat liver slices in the presence of acetate-1-C^{14} (Sodium acetate-1-C^{14}, 5 mmolar, 32,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Concn. of substrate added (mmolar)	$C^{14}O_2$ (c.p.m./mg dry wt. of tissue)	
	Substrate added	
	Sodium acetate	Ethanol
0	93.7	93.7
1.0	82.0	68.4
1.5	77.2	58.0
2.5	68.3	52.6
3.0	65.5	46.8
5.0	52.6	37.4
10.0	43.8	36.9
25.0	22.0	35.4
50.0	10.0	36.9

from 1 mmolar to 5 mmolar a maximum of inhibition is reached and the $C^{14}O_2$ production remains unchanged even when the concentration of ethanol is 50 mmolar. At low concentrations of ethanol, the inhibition is nearly twice as great as that caused by the addition of the same concentrations of unlabelled acetate (Table II).

TABLE II

Percentage inhibition of $C^{14}O_2$ formation from acetate-1-C^{14} (5 mmolar) in presence of rat liver slices by added ethanol and acetate (Sodium acetate-1-C^{14}, 5 mmolar, 32,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Concn. of substrate added (mmolar)	Sodium acetate		Ethanol		Theoretical for isotopic dilution
	Mean	Mean deviation	Mean	Mean deviation	
1	12.5	±3.5	27	±4.8	16.7
1.5	17.5	±4.1	38	±4.1	23.1
2.5	26.9	±3.1	45.8	±4.2	33.3
3	30.3	±4.6	50.2	±6.2	37.5
5	40.5	±6.8	60	±4.6	50
10	53	±7	61	±6	66.7
25	77	±8	62	±7	83.4
50	89	±9	62	±7	91

The depression of $C^{14}O_2$ production may be explained as being either due to inhibition or to isotopic dilution of labelled acetyl-CoA by unlabelled acetyl-CoA derived from ethanol. To examine this possibility, acetate was added to the incubation medium at the same concentration as ethanol and both experiments were carried out at the same time using tissue slices from the same animal. Determinations of the total carbon dioxide were also included in the experimental procedure. It was found, as expected, that addition of acetate results in depression of C^{14}-carbon dioxide production (Table I). When the extent of suppression of $C^{14}O_2$ formation caused by unlabelled acetate, or ethanol, is compared with the theoretical value for isotopic dilution, it is seen that the suppression caused by ethanol at lower concentrations than 5 mmolar is greater than can be accounted for by isotopic dilution. On the other hand, the suppression caused by addition of equivalent quantities of acetate is always (except at concentrations greater than 10 mmolar) significantly lower than the theoretical value for isotopic dilution (Table II). This is doubtless due to the fact that there is an increased rate of oxidation of acetate with increase in its concentration. This may be calculated from the values given in Table I. The fact that the inhibition of $C^{14}O_2$ production in the presence of ethanol is greater than the values for theoretical isotopic dilution, assuming that all the ethanol is converted to acetate or acetyl-CoA, is evidence supporting the conclusion that ethanol inhibits the oxidation of acetate to carbon dioxide in rat liver slices.

This conclusion is confirmed by the fact that in the presence of ethanol the rate of total carbon dioxide formation is also lowered, whereas the addition of the equivalent amounts of acetate has relatively little effect on total carbon dioxide production. Typical results are shown in Table III.

TABLE III
Effects of *n*-alcohols and salts of aliphatic fatty acids on carbon dioxide formation by rat liver slices in presence of 5 mmolar acetate
(Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Concentration (mmolar)	CO_2 formed (μg/mg dry wt. of tissue)			
	With alcohols added		With acids added (as salts)	
Endogenous	17.5±2.4		17.5±2.4	
5 mmolar acetate present	20.2±2.3		20.2±2.3	
Added 2.5	Ethanol	13.2±1.8	Acetic	21 ±2.8
5		10.0±1.6		21.5±2.4
10				19.1±2.3
50		7.4±1.0		17.1±2.3
Added 1	Propanol	12.2±1.9	Propionic	12.8±2.1
2		6.8±1.1		8.8±1.3
Added 1	Butanol	12.6±1.8	Butyric	15.6±2.1
2.5		8.8±1.5		14.6±2.2
Added 1	Pentanol	8 ±1.3	Pentanoic	15.5±2.3
2		5 ±1.0		9.2±1.5

Effects of Other Aliphatic Alcohols and Fatty Acids on $C^{14}O_2$ Formation from Acetate-1-C^{14}

The inhibitory effects of propanol and propionate on $C^{14}O_2$ formation from acetate-1-C^{14} greatly exceed those due to ethanol or unlabelled acetate. The results given in Tables IV and V show that 1 mmolar propanol causes 82% depression of $C^{14}O_2$ activity with a lesser effect on total carbon dioxide production (Table III). It is also seen that the inhibitory effects of propionate are of the same order of magnitude as that due to propanol. The production of total carbon dioxide and $C^{14}O_2$ is inhibited to the same extent by these two compounds.

TABLE IV

Effects of aliphatic alcohols and salts of aliphatic fatty acids on the formation of $C^{14}O_2$ by rat liver slices in the presence of acetate-1-C^{14} (Sodium acetate-1-C^{14}, 5 mmolar, 32,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Concn. of fatty acid or alcohol (mmolar)	$C^{14}O_2$ (c.p.m./mg dry wt. of tissue)			
0.0	Propionic acid	81	Propanol	81
0.25		63		61.1
0.5		50		51.4
1.0		18.6		14.6
2.5		5.7		3.2
0.0	Butyric acid	88.8	Butanol	88.8
0.5		73		66
1.0		65		46
2.5		37		16
0.0	Pentanoic acid	84	Pentanol	84
0.5		59.5		61.6
1.0		29.8		15.8
1.5		16.2		9.0
2.5		6.1		3.3
0.0	Hexanoic acid	77	Hexanol	77
0.5		49		52.5
1.0		41		40.5
1.5		28		20.8
2.5		19.7		13.8

Compared with ethanol and propanol, butanol and butyrate show intermediate inhibitory effects on acetate-1-C^{14} conversion to $C^{14}O_2$. Butanol and butyrate depress the production of both radioactive carbon dioxide as well as total carbon dioxide from acetate. However, the inhibitory effect of butyrate is less than that of butanol. These results on the inhibitory effect of butyrate on acetate-1-C^{14} oxidation are similar to those shown by Masoro *et al.* (9).

The effects of pentanol (Tables IV and V) on acetate oxidation are very similar to those of propanol. Pentanol (2.5 mmolar) produces almost complete suppression of radioactive carbon dioxide production from acetate-1-C^{14}. Pentanoate reduces radioactive carbon dioxide production like propionate

TABLE V

Percentage inhibitions of rates of $C^{14}O_2$ formation from acetate-1-C^{14} in presence of rat liver slices by salts of added aliphatic acids and *n*-alcohols
(Sodium acetate-1-C^{14}, 5 mmolar, 32,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Concn. of fatty acid or alcohol added (mmolar)	Percentage inhibitions			
0.25	Propionic acid	22	Propanol	24
0.5		38		37
1.0		77		82
2.5		93		96
0.5	Butyric acid	18	Butanol	25
1.0		27		52
2.5		58		82
0.5	Pentanoic acid	29	Pentanol	27
1.0		65		81
1.5		80		89
2.5		94		96
0.5	Hexanoic acid	36	Hexanol	32
1.0		47		47
1.5		64		73
2.5		75		82

and it is perhaps a little less effective. At 2.5 mmolar concentration the production of $C^{14}O_2$ in the presence of pentanoate is only 6% of the control.

Hexanol (2.5 mmolar) inhibits production of $C^{14}O_2$ from acetate-1-C^{14} by 82% (Table V). Its inhibitory action is comparable with that of butanol, which at 2.5 mmolar concentration also depresses the production of $C^{14}O_2$ by 82%. Hexanoate (2.5 mmolar) depresses the $C^{14}O_2$ production by 75%, indicating that it influences the oxidation of acetate-1-C^{14} in a manner similar to that of acetate and butyrate. The results show that, with increasing concentration of fatty acids, radioactive carbon dioxide formation is diminished correspondingly. With alcohols, the production of $C^{14}O_2$ is depressed to a somewhat greater extent than with equivalent concentrations of fatty acids.

A fact to be noted from the results given in Table V is that the aliphatic alcohols and acids which have even-numbered carbon atoms are less inhibitory to $C^{14}O_2$ formation from acetate-1-C^{14} than the neighboring odd-numbered aliphatic alcohols and acids.

Combined Effects of Ethanol and Other Alcohols on Acetate Oxidation

Experiments were carried out to discover whether ethanol in conjunction with other alcohols inhibits the respiration of liver slices to a greater extent than when it is used alone. The ethanol was mixed with one of the following alcohols: propanol, butanol, pentanol, isopropanol, isobutanol, and isopentanol.

As can be seen from the typical results given in Table VI the alcohols show additive inhibitory effects. The inhibition of $C^{14}O_2$ production caused by 2.5 mmolar ethanol which amounts to 47% of the control value is increased

TABLE VI

Combined effects of ethanol and other alcohols on the production of $C^{14}O_2$ by rat liver slices in the presence of acetate-1-C^{14} (Sodium acetate-1-C^{14}, 5 mmolar, 32,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Ethanol (mmolar)	Added alcohols (mmolar)	$C^{14}O_2$ Radioactivity (c.p.m./mg dry wt. of tissue)	$C^{14}O_2$ % inhibition
—	—	91	—
2.5	—	48.5	47
2.5	Propanol 0.25	33.4	63
2.5	Propanol 0.50	32.4	64
2.5	Butanol 0.50	35.5	61
2.5	Pentanol 0.50	17.3	81
2.5	Pentanol 1.0	5.6	94
2.5	Isopropanol 0.25	50	45
2.5	Isopropanol 0.50	34.4	62
2.5	Isobutanol 0.50	48.5	47
2.5	Isopentanol 0.50	35.3	61
2.5	Isopentanol 1.00	30.3	67

to 63% by 0.25 mmolar propanol, to 61% by 0.5 mmolar butanol, and to 81% by 0.5 mmolar pentanol. However, when the concentration of propanol or pentanol is further increased, the resulting inhibition does not represent the sum of the depressing effects of each of the two alcohols.

It was also found that neither 0.5 mmolar isobutanol nor 0.25 mmolar isopropanol in combination with 2.5 mmolar ethanol lowers the $C^{14}O_2$ production obtained with ethanol, but that 0.5 mmolar isopropanol with ethanol gives a greater inhibitory effect (62%) than ethanol alone. Isopentanol (0.5 mmolar) with ethanol depresses the $C^{14}O_2$ production by 61%.

Effects of Allyl Alcohol and Tribromoethanol on $C^{14}O_2$ Production

Allyl alcohol is as potent an inhibitor of acetate-1-C^{14} conversion to $C^{14}O_2$ as propanol or pentanol. Allyl alcohol (3 mmolar) depresses the production of $C^{14}O_2$ in rat liver slices from acetate-1-C^{14} by 94% (Table VII).

The effect of tribromoethanol, CBr_3CH_2OH, on rat liver slices in presence of acetate-1-C^{14} is similar to that of ethanol. At 2.5 mmolar concentration it causes 44% inhibition of $C^{14}O_2$ production. However, the fall of $C^{14}O_2$ formation is progressive with increased tribromoethanol concentrations until the radioactivity of the CO_2 ceases when the concentration of tribromoethanol reaches about 20 mmolar. The rates of oxygen consumption of rat liver slices are diminished by both allyl alcohol and tribromoethanol, but the inhibitions of the rates of $C^{14}O_2$ formation from acetate-1-C^{14} exceed those of the rates of respiration (Table VII).

Effects of Aliphatic Alcohols on the Incorporation of Radioactivity from Acetate-1-C^{14} into Proteins and Lipids

As the results of previous experiments have shown that aliphatic alcohols inhibit the production of $C^{14}O_2$ by rat liver slices from acetate-1-C^{14}, as well

TABLE VII

Effects of allyl alcohol and tribromoethanol on the production of $C^{14}O_2$ by rat liver slices in the presence of acetate-1-C^{14} (Sodium acetate-1-C^{14}, 5 mmolar, 32,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Substrate concn. (mmolar)	Q_{O_2}	$C^{14}O_2$	
		Radioactivity (c.p.m./mg dry wt. of tissue)	% inhibition
Allyl alcohol			
0	9.1	84.0	—
3	4.3	5.0	94
6	3.8	2.0	97
Tribromoethanol			
0	8.8	81.5	—
0.5	8.2	67.7	17
1.0	7.5	64.0	21
2.5	6.4	45.5	44
5.0	5.3	27.0	67
10.0	2.2	5.5	93
20.0	1.9	0.2	100

as the total carbon dioxide production, it was of interest to study the effects of these compounds on the incorporation of radioactive carbon from acetate-1-C^{14} into hepatic proteins and lipids. The effects of ethanol, propanol, butanol, pentanol, and allyl alcohol on protein synthesis are recorded in Table VIII.

TABLE VIII

Effect of alcohols on the incorporation of acetate-derived C^{14} into lipids and proteins (Alcohols, 3 mmolar; sodium acetate-1-C^{14}, 5 mmolar, 720,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Alcohols (3 mmolar)	Lipids and alcohol-soluble proteins		Proteins	
	Radioactivity (c.p.m./mg dry wt. of tissue)	% inhibition	Radioactivity (c.p.m./mg dry wt. of proteins)	% inhibition
Control	644 ± 12	—	84.5 ± 8.7	—
Ethanol	423 ± 23	34	57 ± 5	33
Propanol	84 ± 4	87	8.7 ± 2	90
Butanol	222 ± 4	65	17 ± 1.5	80
Pentanol	148 ± 21	77	7 ± 1.9	92
Allyl alcohol	19 ± 2	97	4 ± 0.7	95

In these experiments, rat liver slices were incubated with 5 mmolar acetate-1-C^{14} and the alcohols were added at 3 mmolar concentration. The results show that propanol, pentanol, and allyl alcohol, at this concentration, almost completely suppress the synthesis of labelled proteins; ethanol has about one-third of the inhibitory power of the other alcohols at the concentration tested.

Another pathway of acetate-1-C^{14} metabolism by liver slices, affected by alcohols, is the incorporation of radioactivity into hepatic lipids and alcohol-

soluble proteins. The results (Table VIII) show that out of the five alcohols tested ethanol at 3 mmolar causes the least inhibition (34%). Other alcohols, i.e. propanol, butanol, pentanol, and allyl alcohol, at 3 mmolar inhibit the formation of labelled lipids and alcohol-soluble proteins by over 65%.

The effects of ethanol and sugars on the incorporation of acetate-1-C^{14} into fatty acids in presence of rat liver slices were also investigated (Tables IX, X), using the experimental procedure of Lieber *et al.* (13). It was found

TABLE IX

Effects of ethanol and acetate on incorporation of acetate-derived C^{14} into fatty acids (Sodium acetate-1-C^{14}, 0.29 mmolar, 260,000 c.p.m./vessel. Krebs–Ringer bicarbonate medium, pH 7.4; 95% O_2, 5% CO_2; 3 hours; 37°; 3 ml)

	Radioactivity of fatty acids (c.p.m./mg dry wt. of tissue)			
	Concentration of:			
Control (no extra fatty acid or alcohol added)	Unlabeled sodium acetate added		Ethanol added	
	2.5 mmolar	10 mmolar	2.5 mmolar	10 mmolar
850	553	232	659	294
% inhibition	35	73	23	65

TABLE X

Effects of sugars on the incorporation of acetate-derived C^{14} into fatty acids (Sodium acetate-1-C^{14}, 0.29 mmolar, 200,000 c.p.m./vessel. Krebs–Ringer bicarbonate medium, pH 7.4; 95% O_2, 5% CO_2; 3 hours; 37°; 3 ml)

	Radioactivity of fatty acids (c.p.m./mg dry wt. of tissue)			
Expt.	Control (no sugar added)	Sorbitol, 60 mmolar	Fructose, 60 mmolar	Glucose, 60 mmolar
1	567	500 525	814 778	
2	364 536	270	216	600 1175
3	492	392	300	900 1000
Average	490	420	530	920

that ethanol inhibits the incorporation of radioactivity into fatty acids to about the same extent as, or perhaps a little less than, an equivalent amount of acetate (Table IX). It may be noted that calculations from the results given in Table IX show that with increased acetate concentration there is increased incorporation of acetate-1-C^{14} into the fatty acids. The presence of glucose (60 mmolar) nearly doubles the amount of radioactivity incorporated into fatty acids (see also 15) whereas that of sorbitol or fructose has little or no effect (typical results are given in Table X). These conclusions differ from those of Lieber *et al.* (13), whose results are difficult to assess owing to

the absence of control data giving radioactivities of the fatty acids of the liver slices when they are incubated in the presence of only the trace quantity of acetate-1-C^{14} and in the absence of added 10 mmolar acetate.

Effect of Alcohols on Total Utilization of Radioactivity from Acetate-1-C^{14} by Rat Liver Slices

The results so far recorded show that aliphatic alcohols have pronounced inhibitory effects on acetate-1-C^{14} metabolism in rat liver slices. The following metabolic pathways are affected: (1) $C^{14}O_2$ production; (2) total carbon dioxide production; (3) incorporation of radioactive carbon into proteins; (4) incorporation of radioactive carbon into lipids and alcohol-soluble proteins. The extent of inhibition at a given concentration varies with the structure of the alcohol. Experiments were carried out to discover how far the aliphatic alcohols reduce the utilization of acetate-1-C^{14} when this is incubated with rat liver slices.

The typical results (given in Table XI) show that the alcohols tested depress the utilization of acetate-1-C^{14}. The inhibitive effects of propanol, pentanol, and allyl alcohol, each at 3 mmolar, range between 64% and 95% of the

TABLE XI

Effects of *n*-alcohols (3 mmolar) on utilization of radioactive acetate (Sodium acetate-1-C^{14}, 5 mmolar, 165,000 c.p.m./vessel. Krebs–Ringer phosphate medium, pH 7.4; 3 ml; 1 hour; 37°)

Alcohols	Radioactivity disappeared from incubating medium	
	c.p.m./mg dry wt. of tissue	% inhibition
Control	990 ± 124	—
Ethanol	659 ± 187	33
Propanol	352 ± 16	64
Butanol	554 ± 129	44
Pentanol	52 ± 11	95
Hexanol	263 ± 44	73
Allyl alcohol	108 ± 11	89

control value, whereas the effect produced by ethanol amounts to 33%. It will be seen that there is an alternation in the inhibition caused by alcohols. Alcohols with an odd number of carbon atoms, such as propanol and pentanol, exhibit larger inhibitions than the neighboring even-numbered alcohols, such as ethanol, butanol, and hexanol.

These results may be regarded as confirmatory of those relating to the inhibition by aliphatic alcohols of acetate-1-C^{14} conversion to $C^{14}O_2$ (Table V), or of other aspects of acetate metabolism.

Discussion

The results recorded in this paper, on the effects of aliphatic alcohols and the corresponding acids on the formation of $C^{14}O_2$ from acetate-1-C^{14} by respiring rat liver slices, show that ethanol, at low concentrations, exercises a larger inhibition than can be accounted for if the ethanol is rapidly converted

into acetate with consequent isotopic dilution. At low concentrations (up to 5 mmolar) ethanol is more inhibitory, in the experimental period of time, than an equivalent quantity of acetate in suppressing $C^{14}O_2$ formation. With higher concentrations (> 10 mmolar) the isotopic diluting effect of added acetate exceeds the inhibitory effect of ethanol added at the same concentration. The fact that the inhibitory effect of ethanol reaches a maximum at 5 mmolar (Table II), and is constant up to 50 mmolar, points against a major inhibition due to the alcohol per se, for results (2) have shown that inhibition of tissue respiration by the aliphatic alcohols increases markedly with increase of concentration. Nevertheless, a direct inhibition of acetate oxidation to CO_2, and of endogenous CO_2 formation from liver, by ethanol definitely occurs (Table III). The results, on the whole, are consistent with the conclusion that ethanol *at low concentrations* is more quickly converted into acetyl-CoA than acetate itself and that its speed of conversion into acetyl-CoA reaches a maximum at about 5 mmolar with rat liver slices. There seems to be increasing evidence to indicate that ethanol oxidation is independent of acetate, proceeding through acetaldehyde and acetyl-CoA (19, 20, 21), and it has already been suggested (16) that acetyladehyde is more quickly converted into an active two-carbon intermediate (presumably acetyl-CoA) than acetate in rat liver slices.

n-Propanol is much more inhibitory than ethanol, but its effect is quantitatively identical with that of an equivalent concentration of propionate. This points to the conclusion that both propionate and *n*-propanol are rapidly converted to propionyl-CoA (or methylmalonyl-CoA), which is the effective inhibitor of acetate oxidation by competition with acetyl-CoA. But, again, propanol per se shows direct inhibitory effects on acetate oxidation. *n*-Butanol, *n*-pentanol, and *n*-hexanol have inhibitory effects identical, within experimental error, with those of equivalent concentrations of the corresponding fatty acids. These facts point also to the alcohols and fatty acids being converted into acyl-CoA derivatives which inhibit $C^{14}O_2$ formation from radioactive acetate by competition or isotopic dilution. A point of interest is that, at equivalent concentrations, *n*-butanol is less effective an inhibitor than *n*-propanol or *n*-pentanol and that *n*-pentanol is more effective than either *n*-butanol or *n*-hexanol. These alternations might be explained on the basis of formation of mixtures of acetyl-CoA and the highly inhibitory propionyl-CoA from the long-chain aliphatic alcohols (or fatty acids). Tribromoethanol is much less active than propanol (for equivalent concentrations) in the inhibition of $C^{14}O_2$ from acetate-1-C^{14}, probably due to its lesser rate of oxidation in rat liver slices. Nevertheless it is an effective inhibitor of acetate oxidation. Allyl alcohol is a much more potent inhibitor.

It may be recalled that propionate is a well-known inhibitor of acetoacetate formation in liver slices (12) and that hepatic acetate metabolism is affected by propionate and butyrate (7, 8). It has been pointed out by Avigan *et al.* (6) that inhibitory effects of fatty acids (and other acids such as benzoic or

ethylthioacetic) on metabolism are probably due to their respective acyl-CoA derivatives. Inhibitory effects of propionate, butyrate, and hexanoate on acetate metabolism (9) may be explained in this manner.

In spite of the fact that isotopic dilution, and competition, by products of oxidation of aliphatic alcohols must be an important factor in the suppression of the rate of $C^{14}O_2$ formation from acetate-1-C^{14}, in presence of rat liver slices, estimations of total CO_2, formed from mixtures of acetate and alcohols, indicate that the latter bring about, at increased concentrations, suppressions of acetate metabolism. This suppression is presumably direct by an inhibitory action of the alcohol on the acetate-activating mechanism.

Attention may be drawn to the role of the aliphatic aldehydes during the course of alcohol oxidations in the liver. It is probable that the following process takes place,

$$\text{acetaldehyde} + \text{CoA} + \text{DPN} \rightarrow \text{acetyl-CoA} + \text{DPNH}_2,$$

a conclusion already reached in investigations with rat brain (acetone) powders (DPN, however, not necessarily being specifically involved (17)) and with a bacterial dehydrogenase system (18). Diversion of CoA, towards aldehyde oxidations, from its participation in other, more normal, aspects of cell metabolism may partly account for the interference of alcohol in normal processes of tissue metabolism.

Ethanol inhibits incorporation of radioactivity of acetate-1-C^{14} into fatty acids in rat liver slices during aerobic incubation for 3 hours at 37°. Nevertheless the inhibition is about the same as, and probably a little less than, that due to an equivalent concentration of acetate. The effect of ethanol may once again be explained as being due partly to isotopic dilution by the acetyl-CoA, derived from the alcohol, pooling with radioactive acetyl-CoA undergoing synthesis to radioactive fatty acids. Glucose shows the largest effect in stimulating fatty acid synthesis from acetate (see also 15), fructose is less effective, and sorbitol shows no effect within experimental error.

The aliphatic alcohols inhibit incorporation of radioactivity from acetate-1-C^{14} into liver lipids and proteins, propanol and pentanol showing the largest effects. There is an alternation between the odd- and even-numbered aliphatic chains and the results again point to isotopic dilution, and competition, by the acyl-CoA derivatives (including propionyl-CoA and acetyl-CoA) formed from the alcohols. Allyl alcohol shows the largest inhibitory effects.

The disappearance, or utilization, of acetate by rat liver slices is inhibited by the aliphatic alcohols, an alternation of inhibitory effects between the odd- and even-numbered fatty chains again being observable. This fact points to the inhibition of acetate activation or acetyl-CoA utilization by propionyl-CoA derived from the odd-numbered acids being larger than that due to acetyl-CoA derived from the even-numbered acids.

Whilst the results indicate the rapid conversion in rat liver slices of aliphatic alcohols and aliphatic acids to common intermediates (presumably propionyl-

CoA and acetyl-CoA), the resulting isotopic pooling, or dilution, accounting partly for the inhibitory phenomena described, suppressions by the alcohols of acetate metabolism, possibly directly by competition for the acetate-activating enzyme, evidently also take place.

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STUDIES ON PLASMINOGEN

II. A COMPARISON OF THE ESTEROLYTIC, CASEINOLYTIC, AND FIBRINOLYTIC ACTIVITIES OF BOVINE AND HUMAN PLASMINOGEN[1]

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Abstract

Preparations of bovine and human plasminogen which varied in purity and mode of preparation were activated and assayed for esterolytic, caseinolytic, and fibrinolytic activities. Variations in the ratio of attack of bovine plasmin on TAMe and casein were observed among the plasminogen preparations which represent different stages of purification. Two plasminogen products with different TAMe/casein ratios were separated from both bovine and human products with intermediate TAMe/casein ratios, by the use of phosphate buffer as a precipitant. Bovine plasminogen preparations extracted with acid, then subjected to alkali denaturation, were purified with respect to their caseinolytic activity, but the esterolytic activity remained the same or decreased, resulting in a decrease in the TAMe/casein ratio. Human plasminogen prepared from fraction III by this method showed as much as a fourfold decrease in the TAMe/casein ratio. The observed differences in activity of various plasminogen preparations towards the two substrates, TAMe and casein, may arise from the chemical treatment and manipulation that the proenzyme receives during purification. When the fibrinolytic activity was compared with the caseinolytic activity, a single straight-line relationship was obtained for both bovine and human plasminogen preparations. However, a similar relationship was not found between the fibrinolytic and esterolytic activities.

Introduction

Plasminogen, a component of blood, is the inactive form of the enzyme plasmin, which is responsible for the dissolution of the fibrin clot in vivo. Plasminogen can be activated to plasmin by a variety of activators in vivo, and the enzymatic activity that is developed can be expressed in terms of its action on casein or other proteins, on the fibrin clot, or on synthetic amino acid esters.

In this laboratory, an esterase assay method, based on the hydrolysis of *p*-toluenesulphonyl-L-arginine methyl ester (TAMe) by plasmin, has been used routinely in following the purification of bovine plasminogen, and about the same degree of purification was calculated whether the TAMe esterase or casein hydrolysis method of assay was used (1). However, some variations in the ratio of attack of bovine plasmin on TAMe and casein have been observed among the various plasminogen preparations which represent different stages of purification.

It has also been observed that certain treatments will significantly lower the TAMe/casein ratio. These observations suggest that there may be two pro-

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enzymes in plasminogen preparations or that two separate activities may be formed on activation of plasminogen. Troll, Sherry, and Wachman (2) have reported that the ratio of attack on the two esters, TAMe and lysine ethyl ester (LEe), varied from preparation to preparation of human plasminogen and, on this basis, suggested the presence of more than one plasminogen. More recently, Markus and Ambrus (3) showed that the TAMe esterase activity and caseinolytic activity of streptokinase-activated human plasminogen develop at different rates.

Experimental

Plasminogen Preparations

The bovine and human plasminogen preparations tested are described in Tables I and II, respectively.

TABLE I

Description of bovine plasminogen preparations

Preparation No.	Name	Description
1	Ammonium sulphate ppt.	Bovine serum fraction obtained at 30% ammonium sulphate saturation (4); precipitate dialyzed against water to remove ammonium sulphate, and adjusted to pH 7.4 with 0.1 *N* NaOH
2	Bovine IEP	Preparation 1 subjected to isoelectric precipitation at pH 5.3 and 40-fold dilution according to the work of Dalby, Cole, and Mertz (5); precipitate dissolved in water, adjusted to pH 7.4, and lyophilized
3	DEAE – product A	Preparation 2 chromatographed on DEAE–cellulose column according to Cole and Mertz (1); eluate dialyzed, adjusted to pH 7.4, and lyophilized
4	DEAE – 2× phosphate ppt.	Preparation 3 dissolved in water and plasminogen precipitated twice with potassium phosphate (1); precipitate dialyzed, adjusted to pH 7.4, and lyophilized
5	DEAE – 1× phosphate ppt.	Same as preparation 4 except plasminogen precipitated only once with potassium phosphate (1)
6	DEAE – 1× phosphate supernate IEP	Supernatant remaining after removal of preparation 5 was adjusted to pH 5.4, and the precipitate that formed was dissolved, dialyzed, adjusted to pH 7.4, and lyophilized
7	Bovine IEP – Kline	Preparation 2 was subjected to the Kline method of purification for human plasminogen (6); product dialyzed, adjusted to pH 7.4, and lyophilized
8	DEAE – product B	The method of preparation was the same as for DEAE – product A (preparation 3)
9	DEAE – product B – Kline	Preparation 8 was subjected to the acid–alkali denaturation method of Kline (6) up to and including solution A

TABLE II

Description of human plasminogen preparations

Preparation No.	Name	Description
10	Human blood plasma, fraction III	See footnote *
11	Human IEP	Human IEP was prepared from reconstituted dried pooled human plasma;† the plasma was defibrinated and subjected to ammonium sulphate precipitation and isoelectric precipitation by the same methods used for bovine IEP
12	Human IEP	Prepared from outdated human plasma obtained from local hospital by same methods used for preparation 11
13	Human plasminogen – Roberts	Prepared from fraction III by Roberts method (7), dialyzed, adjusted to pH 7.4, and lyophilized
14	Human plasminogen – Kline – solution A	Prepared from fraction III by the method of Kline up to and including solution A; dialyzed, adjusted to pH 7.4, and lyophilized
15	Human plasminogen – Kline	Prepared from fraction III by Kline method (6), dialyzed, adjusted to pH 7.4, and lyophilized
16	Human plasminogen – Kline	Prepared as for preparation 15
17	Human plasminogen – Kline	Prepared as for preparation 15
18	Human plasminogen – Kline – supernate IEP	The supernatant remaining after removal of preparation 17 was adjusted to pH 5.4, and the precipitate that formed was dissolved, dialyzed, adjusted to pH 7.4, and lyophilized
19	Human plasminogen – Sgouris	See footnote ‡
20	Human plasminogen – Kline	See footnote §

*Fraction III was obtained through the courtesy of E. R. Squibb and Sons and Dr. J. H. Pert of the American Red Cross.
†Obtained from Lt. Col. J. H. Akeroyd of the Walter Reed Army Institute of Research.
‡We are grateful to Dr. J. T. Sgouris, Michigan Department of Health, for this material.
§We are grateful to Dr. D. L. Kline, Yale University, for this preparation.

Methods of Assay

Esterase method.—The esterolytic activity of plasmin was measured by the manometric technique developed by Dalby and Mertz (8). The hydrolysis of TAMe was used as a measure of esterolytic activity. Bovine and human plasminogen preparations were activated with 1% SKHG (streptokinase[3] – human globulin) at 25° for 1 hour in the presence of 0.01 *M* potassium phosphate buffer, pH 7.4. The plasminogen concentration in the activation mixture was adjusted so that 0.2 ml of activation mixture yielded 3–6 esterolytic units in the assay system. At this concentration of plasminogen and activator, maximum activity was developed in 1 hour of activation. A correction for the small amount of plasmin activity due to the SKHG activator was made in calculating the amount of plasmin developed.

[3]Varidase, Lederle Laboratories.

Casein method.—Proteolytic activity of plasmin was determined by measurement of the hydrolysis of casein as described by Remmert and Cohen (9). Bovine and human plasminogen preparations were activated by 0.2 ml of SKHG (8), an amount which was found sufficient for complete activation of plasminogen in 10 minutes. Proteolytic activity was corrected for SKHG activity.

Fibrin method.—Fibrinolytic activity was measured by determining the lysis time of a 0.1% fibrin clot. A 0.2% bovine fibrinogen (90% clottable[4]) solution in 0.01 *M* potassium phosphate – 0.9% NaCl buffer, pH 7.4, was used. Bovine and human plasminogen preparations were activated at the same plasminogen, SKHG, and buffer concentrations as employed in the esterolytic assay. After 1 hour of activation at 25°, the activation mixture was diluted with potassium phosphate – NaCl buffer, pH 7.4, so that 0.2 ml of the diluted mixture yielded 1–2 fibrinolytic units. The final dilution mixture contained 0.01 *M* phosphate and 0.9% NaCl. The diluted activation mixture was placed in an ice bath where the plasmin was stable for at least 1 hour. Two-tenths of a milliliter of the dilution mixture was pipetted into a small test tube (8 mm × 75 mm), 0.2 ml of bovine fibrinogen solution was added, the mixture clotted with bovine thrombin (200 NIH units/ml) on a stirring rod, and the tube placed in a 25° bath. The time from the addition of fibrinogen solution to complete clot lysis was taken as the lysis time. A standard lysis curve was prepared with the use of chloroform-activated bovine plasmin (4), and one unit of fibrinolytic activity was defined as that amount of plasmin which lysed the 0.1% fibrin clot in 20 minutes.

Determination of Protein Concentration

The optical density (absorbance) of the plasminogen preparations, dissolved in 0.1 *N* NaOH, was measured at 280 mμ in a Beckman DU spectrophotometer. Specific activities of plasminogen preparations are given in terms of the yield of plasmin and expressed as TAMe, casein, or fibrinolytic units per unit of optical density. Specific activity is defined as the number of units in 1 ml of a solution which has an optical density of unity.

Results

The assay results obtained on bovine plasminogen preparations are listed in Table III. The table also contains the TAMe/casein ratios calculated from these values. Bovine plasminogen preparations 1–4 represent plasminogen products that are obtained by purification procedures described previously (1, 4, 5). Although the TAMe/casein ratio is variable among these preparations, the over-all purification from the 30% ammonium sulphate precipitate (preparation 1) from serum is 28-fold on the basis of the esterolytic assay and 26-fold on the basis of the caseinolytic assay.

We have attempted many times to purify bovine plasminogen by the Kline

[4]We are grateful to Dr. A. Pappenhagen of Presbyterian St. Luke's Hospital, Chicago, Illinois, for this material.

TABLE III

Esterolytic, caseinolytic, and fibrinolytic activities of bovine plasminogen preparations*

Preparation No.	Name	Specific activity (units/unit optical density)			
		TAMe units	Casein units $\times 10^{-2}$	Fibrinolytic units	TAMe/ casein
1	Ammonium sulphate ppt.	0.6	8	5.0	7.5
2	Bovine IEP	2.1	21	5.9	10.0
3	DEAE – product A	7.4	70	14.3	10.6
4	DEAE – 2× phosphate ppt.	16.9	209	69.5	8.5
5	DEAE – 1× phosphate ppt.	14.0	159	57.5	8.8
6	DEAE – 1× phosphate supernate IEP	3.1	20	12.9	15.5
7	Bovine IEP – Kline	2.2	52	22.6	4.2
8	DEAE – product B	7.0	65	13.5	10.8
9	DEAE – product B – Kline	4.8	100	37.1	4.8

*The values in the table represent single esterase and casein assays of activated plasminogen samples. Fibrinolytic assays were carried out in triplicate on a single activated sample, and the values in the table are averages of the triplicate assays. The difference between the lowest and highest lysis times never exceeded 7% of the average value.

technique (6), and on using an esterolytic assay method (8), the plasminogen product obtained showed a specific activity equal to or lower than the starting material. Thus, it appeared that on the basis of esterolytic activity, purification had not been achieved. This is illustrated by preparation 7. The specific activity in TAMe units was the same as that of the starting material, bovine IEP (preparation 2), but on casein assay a 2.4-fold purification was indicated. This reduced the TAMe/casein ratio from 10.0 to 4.2.

Another example of a change in ratio was observed after Kline treatment (6) of DEAE – product B (preparation 8). Although there was a decrease in the specific activity in TAMe units after treatment of DEAE – product B, an increase of specific activity in casein units was noted. Here the decrease in the TAMe/casein ratio was from 10.8 to 4.8. Ratios in the range of 4 to 5, obtained by subjecting bovine plasminogen to the Kline method of purification (6), are the lowest ratios yet achieved for bovine plasminogen.

The experiments described above might indicate that at least a part of the esterolytic activity found in the bovine IEP and DEAE – product B exists independently of the proteolytic activity and is destroyed by the Kline treatment (6). Since a decrease in the TAMe/casein ratio was noted when DEAE – product A was purified by potassium phosphate precipitation (1), it seemed likely that the supernatant solution remaining after removal of precipitated plasminogen would contain plasminogen activity with a higher TAMe/casein ratio than the starting material. Such an experiment was performed by precipitating plasminogen (preparation 5), with a ratio of 8.8, from DEAE-cellulose column plasminogen (ratio 10.7) by potassium phosphate treatment (1). After removal of the precipitate by centrifugation, the supernatant solution was adjusted to pH 5.4, and the precipitate that formed was removed by centrifugation. The plasminogen precipitated at pH 5.4 had a TAMe/casein ratio of 15.5 (preparation 6).

The isolation of bovine plasminogen preparations 5 and 6, with TAMe/casein ratios of 8.8 and 15.5, respectively, demonstrates that two types of plasminogen can be separated from a plasminogen preparation (DEAE–product) which has a ratio (10.7) intermediate between these two values. The activation characteristics of preparations 5 and 6 were examined more closely by treatment with small amounts of SKHG. Conditions for activation were those used for activation of plasminogen when the esterolytic assay method is employed. The amount of each plasminogen product in the activation tubes was adjusted so that the yields of TAMe units were the same. After addition of SKHG, portions of the activation mixture were removed at 0, 20, 40, 60, and 80 minutes and assayed for esterolytic and caseinolytic activity. In both preparations, esterolytic and caseinolytic activity increased to a peak value at 60 minutes, and, at 80 minutes, decreases in both activities were noted. The esterolytic activity at 20, 40, 60, and 80 minutes' incubation was almost identical for both preparations. However, the plasminogen that was precipitated with potassium phosphate (preparation 5) showed more caseinolytic activity than the one precipitated at pH 5.4 (preparation 6). TAMe/casein ratios for the former were 12.8, 12.5, 12.2, and 15.1 for the 20, 40, 60, and 80 minute values, respectively, with an average ratio of 13.1. Ratios for the latter were 20.8, 21.8, 23.1, and 23.5 at the same activation time periods, with an average ratio of 22.3. Based on these activation and assay data, the pH 5.4 precipitate contains 1.7 times more esterolytic activity than the potassium phosphate precipitate. This agrees well with the value of 1.8 which can be calculated from the data in Table III (preparations 5 and 6).

The assay data on the human plasminogen preparations are listed in Table IV. Fraction III (preparation 10) shows the highest TAMe/casein ratio (5.3) of any human plasminogen preparation examined. Preparations 11 and 12 (human IEP) from human serum show TAMe/casein ratios of 4.3 and 4.6, respectively. These ratios are about one-half of those found for bovine IEP. When fraction III is purified by the method of Roberts (7), which is a modification of the Kline method (6), the TAMe/casein ratio is decreased from 5.3 to 4.3.

Purification of plasminogen from fraction III by the Kline method has always resulted in a decrease in the TAMe/casein ratio. When the Kline procedure is carried out up to and including solution A, the TAMe/casein ratio is reduced to 2.2, and when solution A is treated with sodium phosphate buffer, the TAMe/casein ratio of the precipitated plasminogen is reduced further. The highest ratio found for human plasminogen purified by the Kline method was 3.0, but in all other preparations examined, the ratio has been in the range of 1.6 to 0.96. The human plasminogen product (preparation 20) sent to us by Dr. D. L. Kline is the most potent which has yet been reported and showed a ratio of 0.96. This suggests that the lower limit of this ratio may be near 1.0 for Kline plasminogen.

A partial separation of the caseinolytic and esterolytic activities of certain human plasminogen preparations can also be made. Human plasminogen (preparation 14), obtained by the Kline method (6) up to and including solution A,

TABLE IV

Esterolytic, caseinolytic, and fibrinolytic activities of human plasminogen preparations*

Preparation No.	Name	Specific activity (units/unit optical density) TAMe units	Casein units $\times 10^{-2}$	Fibrinolytic units	TAMe/casein
10	Human blood plasma fraction III	0.8	15	8.0	5.3
11	Human IEP	0.9	21	10.0	4.3
12	Human IEP	1.7	37	15.2	4.6
13	Human plasminogen – Roberts	3.2	75	23.1	4.3
14	Human plasminogen – Kline – solution A	2.9	132	35.0	2.2
15	Human plasminogen – Kline	5.9	197	61.9	3.0
16	Human plasminogen – Kline	4.1	252	79.3	1.6
17	Human plasminogen – Kline	3.6	240	76.0	1.5
18	Human plasminogen – Kline – supernate IEP	5.2	203	63.8	2.6
19	Human plasminogen – Sgouris	4.7	362	—	1.3
20	Human plasminogen – Kline	6.6	690	—	0.96

Same as footnote, Table III.

has a TAMe/casein ratio of 2.2. When such a solution was treated with sodium phosphate buffer (6), the precipitate (preparation 17) had a ratio of 1.5. The supernatant solution was then adjusted to pH 5.4, and the precipitate that formed (preparation 18) had a ratio of 2.6.

Since the natural function of plasmin is to promote fibrinolysis, we compared fibrinolytic activity of the plasminogen preparations with caseinolytic and

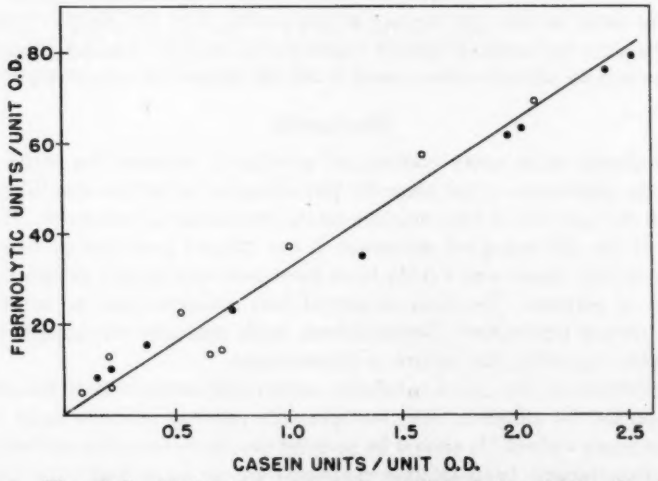

FIG. 1. The relationship between caseinolytic and fibrinolytic activities of bovine and human plasminogen (○ bovine plasminogen; ● human plasminogen).

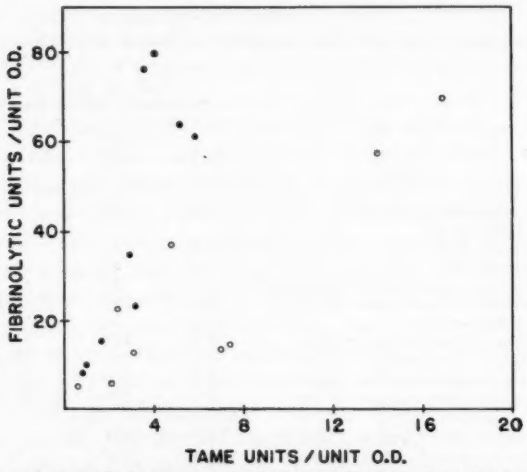

FIG. 2. The relationship between esterolytic and fibrinolytic activities of bovine and human plasminogen (○ bovine plasminogen; ● human plasminogen).

esterolytic activity. The results for the bovine and human products prepared in this laboratory are shown in Tables III and IV, respectively. When these data are used to plot caseinolytic and fibrinolytic specific activity units as abscissae and ordinates, respectively (Fig. 1), a straight-line relationship in which $X = 0.03Y$ is obtained for bovine and human plasminogen preparations. However, when esterolytic and fibrinolytic specific activity units are plotted in the same manner, there is a greater scatter of points, and no single line can be drawn for both bovine and human preparations (Fig. 2). Figure 1 also illustrates that, on the basis of specific caseinolytic activity, human plasmin is as potent as bovine plasmin when using a bovine fibrin clot as substrate.

Discussion

In the choice of an assay method for an enzyme system, the activity of an enzyme on substrates other than its physiological substrate should correlate well with the activity of that enzyme on its physiological substrate. However, the use of the physiological substrate is not always practical or convenient. In recent years, casein and TAMe have been used extensively as substrates for the assay of plasmin. The data presented here indicate that the two methods are not always equivalent. Nevertheless, both methods contribute valuable information regarding the nature of plasminogen.

One objection to the use of synthetic amino acid esters is that the substrate is not specific for plasmin, and non-plasmin esterase activity may produce erroneous assay values. It should be pointed out, however, that the bovine and human plasminogen preparations examined by us have had very little free esterolytic activity before activation, and this, together with the activity due

to the SKHG activator, is subtracted from the final value. All of the assay data thus represent activity which is derived by the action of SKHG on the proenzyme (or proenzymes).

The observed differences in activity of various plasminogen preparations towards the two substrates, TAMe and casein, may well arise from the chemical treatment and manipulation the proenzyme receives during purification. We have noticed with bovine plasminogen, as has Alkjaersig (10) with human plasminogen, that the product purified by the Kline method (6) is less soluble at neutral pH than that prepared by less drastic treatment. For example, bovine plasminogen purified by DEAE-cellulose column chromatography (1) is usually soluble in distilled water at neutral pH at a concentration of 5 mg/ml. The Kline treatment (6) of this chromatographic product reduces the solubility of the plasminogen, and at the same time reduces the ratio of esterolytic to caseinolytic activity.

Markus and Ambrus (3) found that streptokinase activation of human plasminogen results in a non-parallel development of caseinolytic and esterolytic activities. Caseinolytic activity developed rapidly and was followed by a slower development of esterolytic activity. In our activation experiments with bovine plasminogen, however, the time course for appearance of caseinolytic and esterolytic activities was the same.

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SYNTHESIS OF CELLULOSE FROM ETHANOL-SOLUBLE PRECURSORS IN GREEN PLANTS[1]

J. Ross Colvin

Abstract

The formation of microfibrillar material from filtered 80% (v/v) ethanol extracts of etiolated *Avena* coleoptiles and *Pisum* seedlings was demonstrated by electron microscopy. The microfibrils were resistant to alkali, molar acetic acid, and extraction by lipid solvents. After acid hydrolysis, only glucose was detected as a component neutral sugar. The X-ray diffraction powder diagram of these microfibrils was identical with that of a standard alkali-digested bacterial cellulose. On this evidence, the microfibrils are assumed to be cellulose produced from a soluble precursor together with an extracellular bacterial enzyme. The general similarity between these results and those previously obtained with extracts of *Acetobacter xylinum* is discussed.

Introduction

Until recently, published descriptions of cell-free systems capable of forming cellulose microfibrils were limited to homogenates or fractions of homogenates that had been supplemented by adenosine triphosphate or uridine diphosphate glucose (1). However, two reports have now appeared describing the synthesis of cellulose from ethanol extracts of bacterial cultures (2, 3) and one of these extracts has been fractionated to isolate the precursor of bacterial cellulose (4, 5). The purpose of this paper is to report a qualified extension of similar methods to extraction of the precursor of cellulose from green plants, particularly pea seedlings.

Materials and Methods

Plant Materials

Peas (*Pisum sativum;* variety, Arthur) and beans (*Phaseolus vulgaris;* variety, Black Pencil Pod) were germinated in moist vermiculite and grown in darkness for 7 days. The shoots of both were then about 15 cm long. Oat seedlings (*Avena sativa;* variety Lanark) were germinated on moist filter paper and grown in darkness for 5 days before harvest. At this time the coleoptiles were 5–8 cm long.

Method of Extraction

The seedlings or coleoptiles were cut into approximately 2-cm lengths and dropped into sufficient absolute ethanol that the final concentration approached 80% by volume. When all the material was harvested (300–400 g wet weight) the specific gravity of the suspension was adjusted to 0.864 (80% by volume) and the mixture homogenized in a Waring blender. After 1 hour, the tissue

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fragments were removed by centrifugation followed by filtration. In earlier, smaller-scale preparations the supernatant from centrifugation at 15,000 *g* for 15 minutes was passed through a sintered glass bacterial filter, Corning 60 U.F., in order to remove all traces of residual cellulose microfibrils and other cellular debris. In later, larger-scale preparations, where filtration of 2 to 4 liters of aqueous ethanol through the above-mentioned filter proved impractical, the following procedure was adopted: Coarse particulate material was removed by centrifugation at 3000 *g* for 15 minutes and the supernatant was passed through a Corning medium-sintered glass filter to remove smaller particles. The filtrate then passed through a Corning fine-sintered glass filter (not 60 U.F.) to remove the last traces of cellular debris. Control experiments using a second 80% ethanol extraction of the previously extracted tissue fragments, which had been incubated at room temperature in water for 12 hours, demonstrated that no previously formed microfibrils passed the final filter in either of the above procedures. Following the last filtration, 10 ml of either a filtered supernatant of an active culture of *Acetobacter xylinum* (2) or of a filtered water extract of an homogenate of the plant material was added to the ethanol solution from 300 to 400 g wet tissue to supply an enzyme source (2). The enzyme source is added to the filtrate before removal of the ethanol in order to ensure that the cellulose-synthesizing enzyme is present whenever the solution becomes predominantly aqueous. Previous experiments with the bacterial enzyme have indicated that its action is reversibly inhibited by high concentrations of ethanol (2). The resulting mixture was concentrated in 1-liter, round-bottomed flasks to a thick syrup on a rotary evaporator at 40° C. To the syrup in each flask was added 10 ml of water and the slurry incubated at 35° C for 30 minutes to ensure maximal formation of the microfibrillar material.

During all manipulations, extreme care was taken to exclude stray lint, dust, or other microfibrillar contamination from the material in the flasks. All vessels were rinsed before use with redistilled absolute ethanol and kept closed with aluminum foil.

Filtered supernatants of plant homogenates were prepared as follows. Plant tissues were homogenized in a Waring blender for 1 minute in approximately 10 times their weight of water. The suspension was centrifuged at 15,000 *g* for 15 minutes and the supernatant then filtered through a sintered glass bacterial filter, Corning 60 U.F. The filtrate was used immediately.

Extraction and Digestion of Residues from Incubated Extracts

Following incubation of the aqueous suspension of residues from the extract, 40 ml of redistilled absolute ethanol was added to each flask, plus 20 ml of chloroform, and the mixture gently swirled for 15 minutes. This suspension was centrifuged for 15 minutes at 15,000 *g* to yield two fractions of semisolid material, one which floated and the other which sedimented. The floating material and the two liquid phases were poured off and discarded. The heavier residues were suspended in 20 ml of 4% NaOH, digested in boiling water for

10 minutes (2),* diluted with water, and centrifuged as above. After the alkaline supernatant was discarded, the pellet was resuspended in 5 ml of 1 *M* acetic acid which dissolved a large fraction of the residues, and again recentrifuged. Conversion of any carboxylate ions in the solid material to the acid form during this step facilitated its dissolution in a second similar series of extractions by 80% ethanol, hot 4% NaOH, and molar acetic acid. This second series of extractions is essential to remove a fraction of the lipids and noncellulosic polymers which are not completely extracted by the first series.

Electron Microscope and X-Ray Diffraction Examination of Residues

Following the final extraction by molar acetic acid, the insoluble residue was dispersed in water. Drops of the dispersion were placed on formvar films floating on 1% formaldehyde and allowed to stand overnight to remove traces of soluble substances by dialysis through the formvar film; the portion of the film carrying the drop was then mounted over copper electron microscope grids and dried over anhydrous $CaSO_4$. The dried grids were shadowed with Pd–Au (60:40) at an angle of 15° and examined in an RCA EMU I electron microscope.

X-Ray diffraction examinations were carried out with a Hilger–Watts microfocus instrument with samples mounted in a capillary tube in a North American Philips microcamera. With nickel-filtered copper $K\alpha$ radiation, the exposure time was 90 hours at 40 kv and 350–400 μamp.

Hydrolysis of the cellulose samples was by a modification of the method of Croon *et al.* (6). The cellulose was dissolved in 0.025 ml of 72% H_2SO_4 and allowed to stand at room temperature for 30 minutes. Water (0.125 ml) was added to bring the acid concentration to 12% and hydrolysis was completed by heating the solution in a sealed tube at 100° C for 4 hours. The acid was neutralized with washed Amberlite IR-45 (OH) ion-exchange resin (Rohm and Haas) and the aqueous solution taken to dryness on a rotary evaporator at 40° C. The residue, taken up in water, was placed on a chromatographic paper with 2 μg standard glucose. The paper was developed by the descending technique in butanol – acetic acid – water (4:1:1) for 10 hours. Reducing sugars were detected by alkaline silver nitrate (7).

Results

Typical photographs of microfibrillar material found in residues of the ethanol extracts of *Pisum* seedlings and *Avena* coleoptiles are shown in Figs. 1 and 2. Qualitative comparison showed that *Pisum* was much the better source of this microfibrillar, acetic-acid- and alkali-resistant, fat-solvent-insoluble substance. Preliminary experiments with similar extracts of sugar beet leaves (*Beta vulgaris;* variety Klein Wanzleben) indicated that a smaller quantity of a similar material might be obtained from this plant. Repeated attempts to

*Trial experiments showed that the weight loss from a sample of standard bacterial cellulose, which was extracted by hot 4% NaOH, was slightly greater than the weight loss of a similar aliquot extracted by cold 17% NaOH. The hot 4% NaOH treatment therefore is, if anything, a little more rigorous than the classical procedure.

demonstrate a microfibrillar substance from ethanol extracts of *Phaseolus* failed completely, although the plants are larger with a higher growth rate. For these reasons, *Pisum* was selected as the source of material for experiments to be described hereafter.

Acid hydrolysis of the three times water-washed insoluble residues from the ethanol extracts of the pea seedlings, followed by paper chromatography, demonstrated only glucose as a component neutral sugar.

Furthermore, X-ray diffraction examination of samples of the microfibrillar insoluble residues gave, after 90 hours' exposure, a faint but definite powder diagram corresponding to samples of standard cellulose I from bacteria (Table I). The pattern itself is not given because, with the amounts of sample present, three of the five reflections recorded on the negative were too faint to reproduce satisfactorily.

TABLE I

Comparison of X-ray powder diagrams for standard bacterial cellulose I and extracted *Pisum* residues

Obs. ring diameter (cm)		Relative intensities		
Std. cellulose	*Pisum* residues	Std. cellulose	*Pisum* residues	Spacing, Å
0.74	0.74	M	M	6.4
0.86	0.86	M	M	5.5
1.07	1.1	W	W	4.5
1.20	1.22	S	S	4.1
1.98	2.05	W	VW	2.6

An attempt was made to substitute a filtered water extract of homogenized pea seedlings for the filtered supernatant of an active culture of *Acetobacter xylinum* but without success. No microfibrillar material has as yet been seen in any of these preparations.

The total amount of cellulose recovered from an extract of 300–400 g of fresh pea seedlings was never more than a milligram and frequently less. In several preparations, particularly those from *Avena*, only the resolution provided by the electron microscope for microfibrillar material permitted a reliable identification of a fraction of the insoluble material as cellulose. An appreciable proportion of the insoluble residues in all experiments was obviously non-fibrillar and apparently amorphous in appearance (Figs. 1*a*, 1*b*, and 1*c*); certainly not typical microfibrillar cellulose. The nature of this amorphous material is unknown but it resisted repeated applications of the drastic digestion procedure outlined above.

With plant material, preliminary experiments indicated a source of error which may not be immediately evident. If lipid solvents are omitted from the extraction procedure much material will remain, after extraction with alkali and acid, which is microfibrillar in appearance and difficult to distinguish from cellulose by this technique alone. The material does not remain after extraction with 2:1 ethanol–chloroform or 80% ethanol and is presumed to be some form

PLATE I

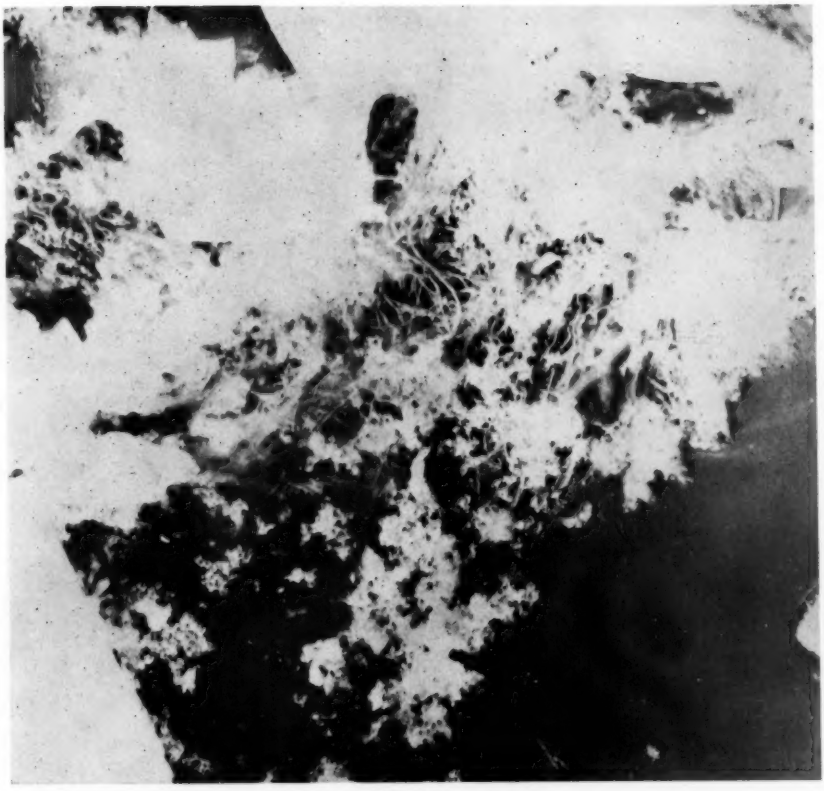

FIG. 1*a*.

FIG. 1. Cellulose microfibrils from 80% ethanol extracts of *Pisum* seedlings plus filtered supernatant of *Acetobacter xylinum* suspensions.

(*a*, Plate I) Tangled clumps of microfibrils. Note also the substantial fraction of non-fibrillar, apparently amorphous material.

(*b*, Plate II) Bundle of microfibrils, again with amorphous insoluble residues. Note left-hand twist of bundle.

(*c*, Plate III) Tangled mass of microfibrils, which are less well associated to form bundles than in (*a*) or (*b*).

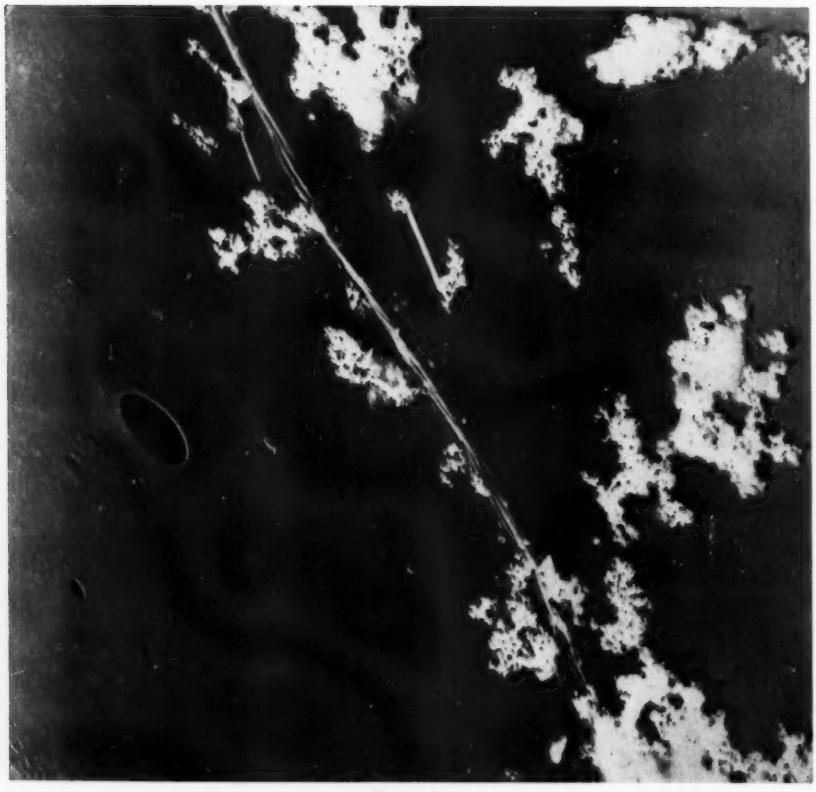

FIG. 1*b*.

PLATE III

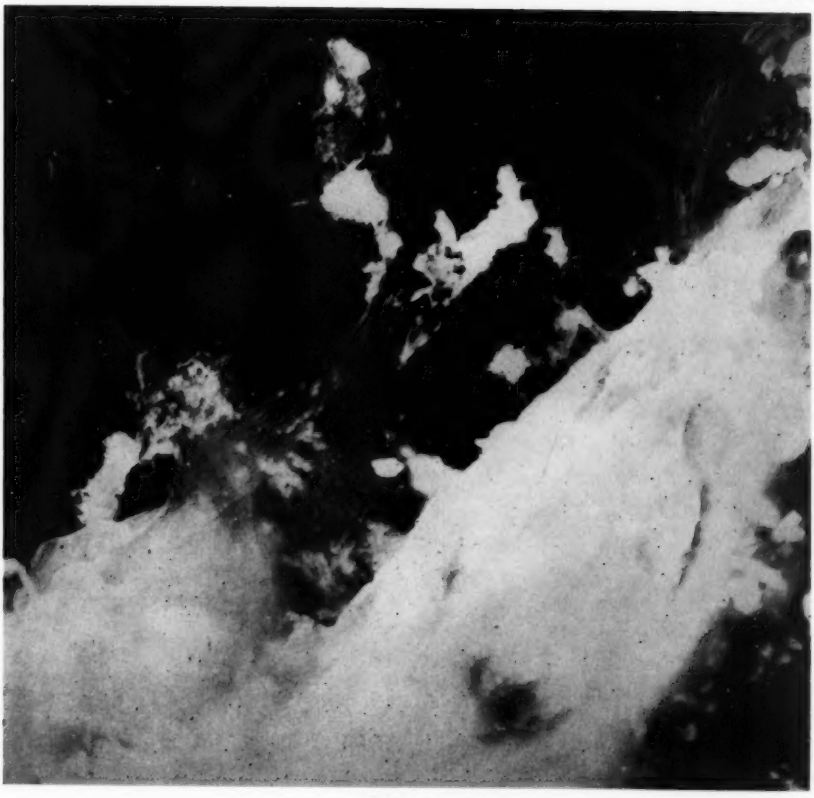

FIG. 1*c*.

FIG. 2*a*.

FIG. 2. Cellulose microfibrils from 80% ethanol extracts of *Avena* coleoptiles plus filtered supernatant of *Acetobacter xylinum* suspensions.
(*a*, Plate IV) Two large bundles of microfibrils.
(*b*, Plate V) Small bundle of microfibrils.
(*c*, Plate VI) A very large bundle of microfibrils from which smaller strands or single microfibrils branch.

PLATE V

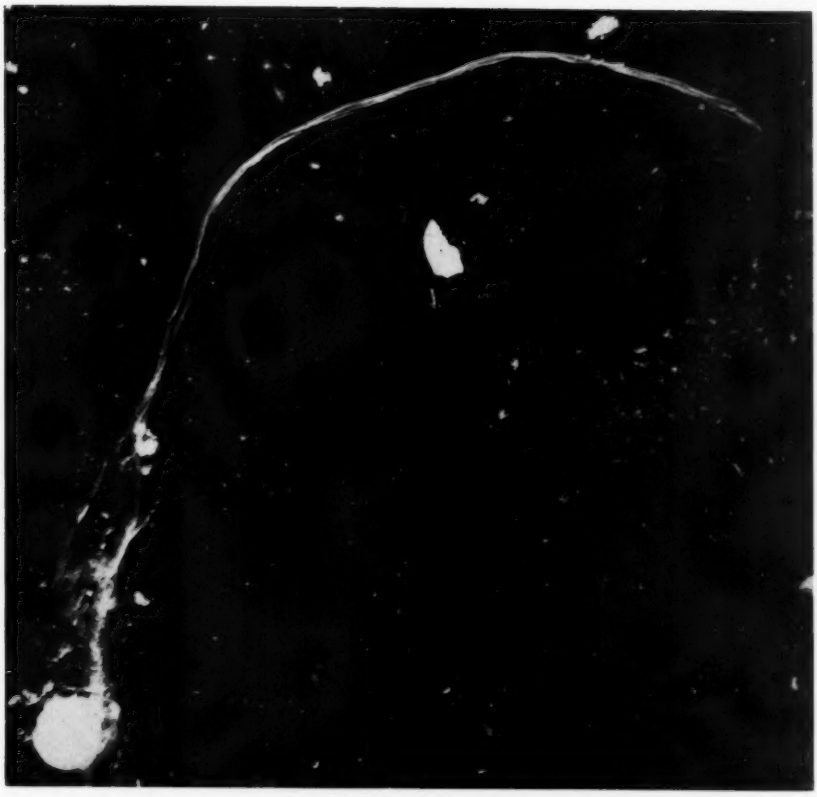

FIG. 2*b*.

FIG. 2*c*.

of lipid, many of which are known to form threads or fibrils under appropriate conditions (8).

Discussion

The alkali resistance, X-ray diffraction pattern, and the glucose derived from hydrolysis of the microfibrillar material are strong evidence that the residues from the ethanol extracts are at least partially composed of cellulose. That the residues are not derived from cellulose formed prior to extraction of the tissues is shown by the following facts.

(*a*) A second 80% ethanol extraction of the plant material yielded no microfibrillar material. If the residues were derived from previously formed microfibrils which had passed the final filtration, a second extraction should have yielded another portion of the same appearance as the first.

(*b*) *Phaseolus* extracts consistently gave no insoluble microfibrillar material, although the amount of original plant tissue was somewhat larger.

(*c*) Ethanol extracts of peas supplemented by filtered water extracts of the same plant yielded no insoluble microfibrillar material.

Consequently, the small residue of resistant microfibrils must have been formed from a precursor (or precursors) present in the filtered ethanol extract during the incubation with an enzyme from *Acetobacter xylinum*. This precursor is extracted from the cytoplasm and the cell walls of the actively dividing and elongating tissues by the rapid immersion in concentrated ethanol. The high ethanol concentration inhibits polymerization of the precursor which takes place rapidly in analogous aqueous systems (9). Presumably, no microfibrils are formed from the pea ethanol extract plus filtered water extract of peas because a water-soluble cellulose-synthesizing enzyme was not released from the tissue under the conditions used. The extremely low yield of cellulose in the successful experiments, even as compared with bacterial cellulose, is consistent with the fact that only the precursor in transit in the thin layer between the cytoplasm and the areas of deposition within the wall may be extracted. Since the volume of this layer is a small fraction of the total cell volume and since, by analogy with bacterial cellulose (9), the steady-state concentration of the plant cellulose precursor may be very low within this small volume, the final yield from hundreds of grams of fresh tissue may easily be vanishingly small. No explanation of the failure to obtain any such precursor from *Phaseolus* can be given at present.

Because the same methods successfully remove the precursor(s) of cellulose from actively metabolizing suspensions of *Acetobacter xylinum* and *Acetobacter acetigenum* and from the tissues of some green plants, the compounds extracted may be similar (and possibly are identical). Furthermore, these results are consistent with the view that the general mechanism of formation of cellulose microfibrils in green plants is similar to that found in bacteria (9, 10). The low molecular weight precursor may be formed in the cytoplasm, transported across the cytoplasmic membrane into the cell wall, and there the glucose residue is

transferred enzymatically to the tip of an elongating microfibril. The non-glucose portion of the precursor may then return to the cytoplasm to be recycled.

The successful substitution of an enzyme from *Acetobacter xylinum* for the native pea seedling enzyme in the formation of cellulose microfibrils suggests that either the two substrates are identical or specificity is limited to the glucose part of the molecule. These experiments, of course, give no evidence for the participation of a primer in cellulose synthesis in green plants but also do not exclude this possibility.

Acknowledgments

The author wishes to thank Dr. G. A. Setterfield, who first urged that these studies be undertaken, following the successful results with *A. xylinum*. The painstaking technical assistance of Mr. L. Sowden is gratefully acknowledged.

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NOTES

A CLOSED CIRCUIT TECHNIQUE FOR OBTAINING GAS SAMPLES FROM A SEALED CHAMBER

W. R. INCH* AND J. A. MCCREDIE†

In the measurement of gaseous metabolism, samples of gas for analysis need to be aspirated from the closed chamber containing the animal. This is often done by inserting a needle attached to a greased hypodermic syringe through a rubber membrane in the wall of the vessel. A small amount of room air can enter the syringe and cause an error in the measurement or an exchange of gases through the punctures in the rubber membrane may eventually alter the concentrations in the chamber. These sources of error have been eliminated by using a mercury siphon (Fig. 1A) to withdraw the gas. A polyethylene tube (1 mm I.D.) with heat-expanded ends and a tygon tube (6 mm I.D.) are joined by a pyrex glass reduction union. The free end of the polyethylene tube is passed through a small hole in the chamber wall and sealed in position, while the other end of the tygon tube is connected to the side arm of a gas analyzer located at a lower level. The system is filled with mercury to a point several centimeters beyond the glass union. A volume of gas is removed from the chamber by manually forming an S-shape in the tygon tube and slowly raising this above the mercury level in the analyzer; the tube is then lowered and the trapped gas rises through the mercury toward the analyzer. A sample for analysis can be transferred in approximately 25 seconds, assuming the first gas drawn, which represents "dead space" in the small tube, is allowed to escape. Another form of the apparatus (Fig. 1B) can be operated more rapidly. Part of the large tygon tube is replaced by a pyrex U-tube of the same diameter, which forms permanently one-half of the S-shape. The U-tube is mounted on a slide constrained to move in the vertical direction, and aspiration and transfer of a gas sample are accomplished by manipulations similar to those previously described.

This apparatus has been used over a number of years with a Fry analyzer (1) to obtain gas from a rat metabolic chamber for oxygen and carbon dioxide determinations (Fig. 2). The method has been employed in another study to withdraw gas from a partially evacuated chamber (2). It is particularly useful in such an experiment because an appropriate adjustment in the relative heights of the mercury columns in the tubes will compensate for chamber pressures either above or below normal atmospheric pressure.

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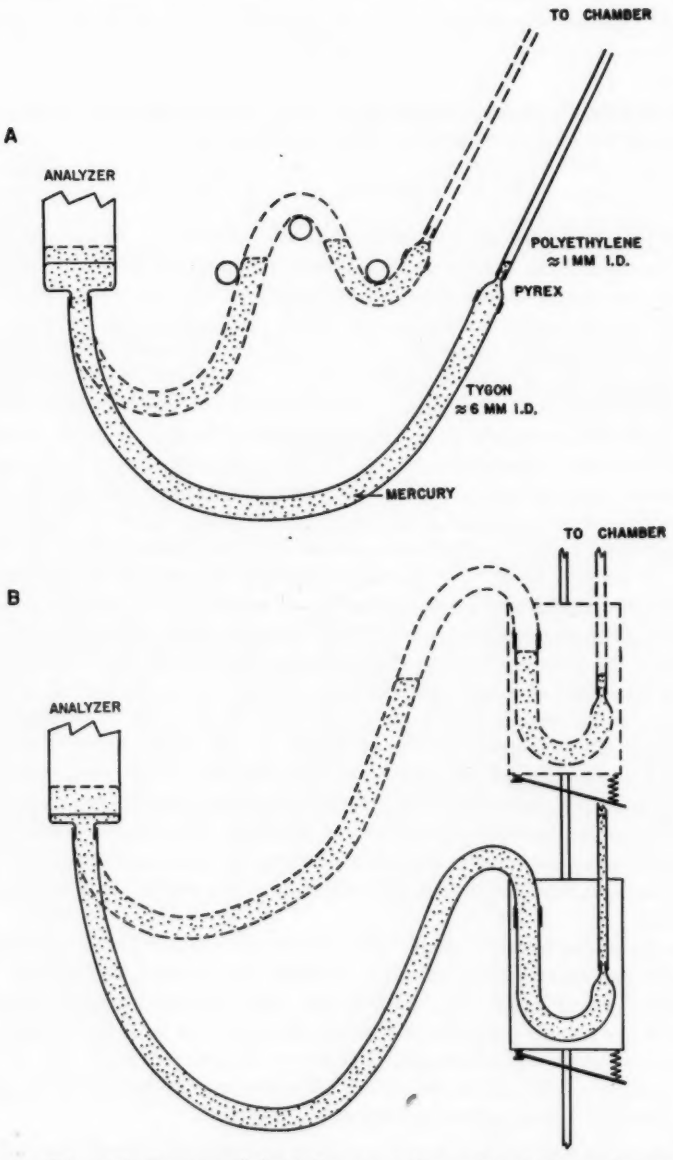

FIG. 1. Schematic diagrams of the siphon apparatus used to obtain gas from a sealed chamber: A, flexible tube; B, U-tube trap.

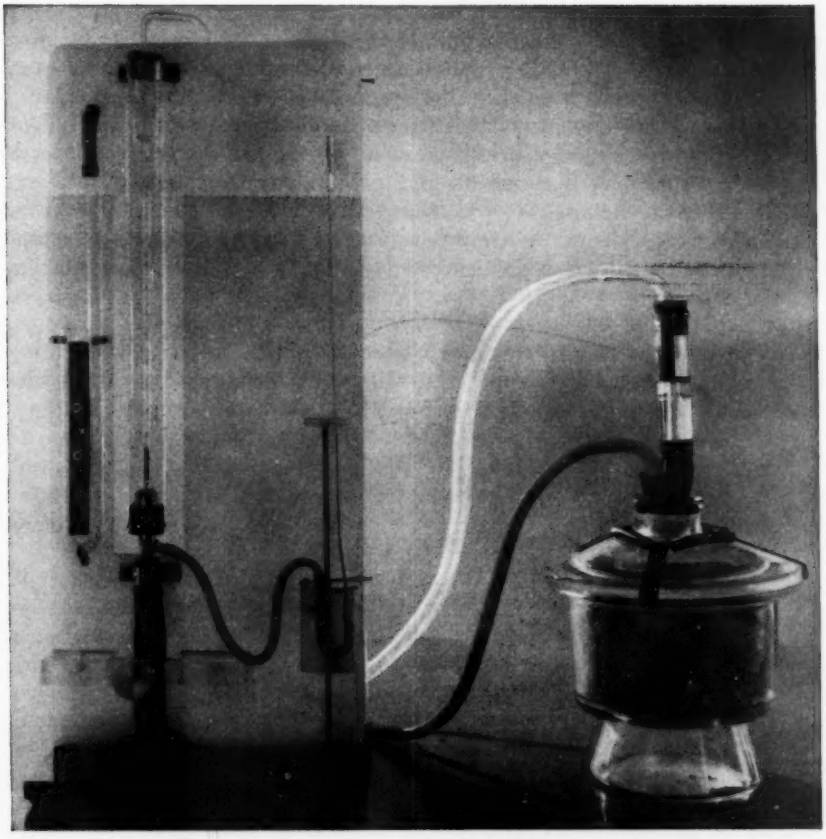

FIG. 2. Apparatus connected between a rat metabolism chamber and a Fry gas analyzer.

There are several factors which must be considered in the application of the above method. The most important is that the gas analyzer should be located near the chamber in order to keep the total length of tubing short. For convenient operation the length of the large tube should not exceed 50 cm while the length of the small one should be less than

$$(D_1/D_s)^2 \bar{L}_1 N.$$

In this expression, D_1 and D_s represent the diameters of the large and small tube, L_1 is the mean length of a number of gas samples siphoned into the large tube, and N the number of samples that must be expelled before drawing the

test sample. If $D_1/D_2 = 6$, $\bar{L}_1 = 5$ cm, and $N = 1$ then the small tube length should be less than 180 cm. The "dead space" in the small tube can be eliminated if the mercury level in the analyzer can be raised and lowered; a much longer polyethylene tube or just the tygon tube can then be used. Only a limited number of chambers can be permanently connected to one analyzer unless a disconnect point near the chamber is provided and this modification has not been completely satisfactory. Finally, mercury exposed to air forms an oxide layer at the interface which can lead to blockage of the polyethylene tube. The mercury must therefore be replaced every 2 to 3 months depending on how often the apparatus is used.

Summary

Gas for analysis may be siphoned from a closed chamber by joining it to a gas analyzer with a flexible tube partly filled with mercury. This method has been found rapid and free from errors due to gas leaks.

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DECARBOXYLATION OF γ-HYDROXYGLUTAMATE TO α-HYDROXY-γ-AMINOBUTYRATE IN RAT BRAIN

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Some years ago, Virtanen and Hietala (1) reported that γ-hydroxyglutamate isolated from *Phlox decussata* was decarboxylated to α-hydroxy-γ-aminobutyrate by a B_{12} vitamin requiring mutant of *Escherichia coli*. γ-Hydroxyglutamate is now known to be a normal intermediate in the breakdown of L-hydroxyproline (2, 3, 4, 5). It was of interest to find out if the former amino acid could be decarboxylated to α-hydroxy-γ-aminobutyrate in rat brain tissue, just as glutamate is decarboxylated to γ-aminobutyrate (6, 7, 8). We wish to report here the results of experiments carried out in vitro, which furnish evidence of decarboxylase activity in rat brain tissue toward γ-hydroxyglutamate. However, no such activity could be found in liver.

Crude homogenates of rat brain or liver were prepared by pulverizing in a glass Potter apparatus 1.5 g of fresh tissue in a cold mixture of 5 ml K_2HPO_4, 0.05 *M*, and 1 ml NaH_2PO_4, 0.05 *M*. Incubation media were prepared by dissolving in each homogenate 250 μg of pyridoxal phosphate, 5 mg of γ-hy-

droxyglutamate-5-C^{14} (epimeric mixture), and finally 5 mg of α-hydroxy-γ-aminobutyrate added as carrier. Following a 2-hour incubation at 38°, the reaction was stopped by adding two volumes of ethanol to each incubation medium, and heating in a water bath for 15 minutes. The precipitated proteins were removed by filtration and the filtrate was concentrated to about 8 ml. The solution was placed on a column (2.5×45 cm) of Dowex 1X4-acetate (200–400 mesh) followed by 1 *N* acetic acid which eluted α-hydroxy-γ-aminobutyrate and glycine immediately, with γ-hydroxyglutamate descending more slowly through the column. Fractions 1–12 (15 ml each) of the effluent, containing the monoamino–monocarboxylic acids, were pooled and evaporated to a few milliliters. Samples were chromatographed in one dimension on Whatman No. 1 paper, using as solvent systems either phenol–water (4:1) or ethanol – acetic acid – water (8:1:1). α-Hydroxy-γ-aminobutyrate was thus easily separated from glycine-1-C^{14} which was formed normally by amination of glyoxylate-1-C^{14} (9), the latter substance resulting from dealdolization cleavage of γ-hydroxyglutamate-5-C^{14} between carbon atoms 3 and 4. The formation of radioactive glyoxylate was qualitatively ascertained by the carrier method and radioactivity measurement of the purified 2,4-dinitrophenylhydrazone.

The results of our experiments are graphically depicted in Figs. 1 to 4 which show the net radioactivity patterns along the paper chromatograms. We were able to characterize by admixture paper chromatography and ninhydrin color test each amino acid responsible for a major peak. Figures 1 and 2 are graphic representations of radiochromatograms obtained in the first series of experiments with rat brain tissue homogenates. Both radiochromatograms indicated that α-hydroxy-γ-aminobutyrate contained a significant amount of radiocarbon. In control incubations lacking only pyridoxal phosphate, decarboxylase activity was considerably reduced if not found negligible. This is the first demonstration of decarboxylase activity in rat brain toward γ-hydroxyglutamate. Furthermore, the presence of a substantial amount of radioactive glycine in the incubated media provides indirect evidence that γ-hydroxyglutamate-5-C^{14} is broken down by dealdolization in the brain tissue.

This latter reaction was found to occur more extensively in rat liver tissue, as judged from the radioactivity of glycine on the chromatograms graphically depicted by Figs. 3 and 4. But the most interesting fact is that on these chromatograms, no radioactivity whatsoever was found to coincide with the ninhydrin spots due to the α-hydroxy-γ-aminobutyrate (at 13–14 cm or R_f 0.56 for Fig. 3; at 17–18 cm for Fig. 4) added as carrier.

The studies described in the present communication justify the conclusion that there exists in rat brain, but not in liver, a decarboxylase which acts upon γ-hydroxyglutamate to form α-hydroxy-γ-aminobutyrate. The important question as to whether γ-hydroxyglutamate is decarboxylated by glutamic acid decarboxylase or by a different decarboxylase is still open.

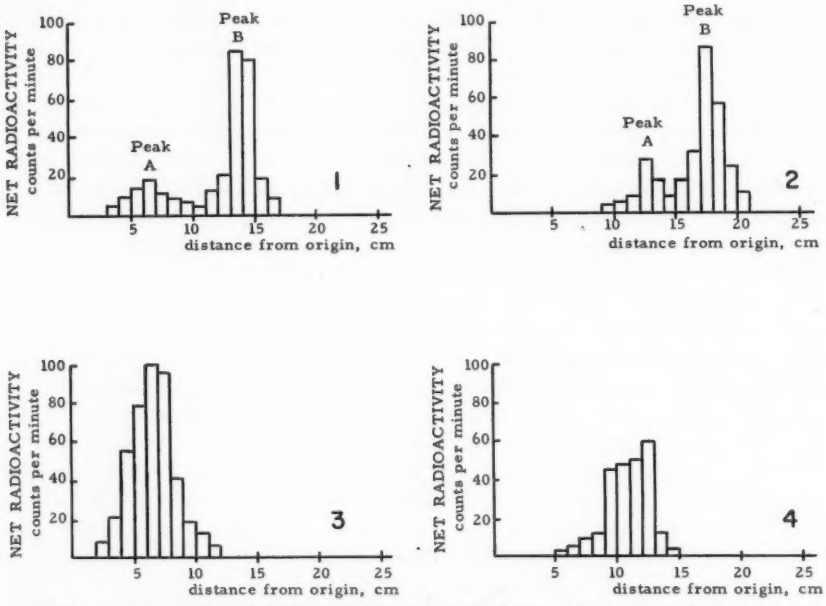

FIG. 1. Ascending paper chromatography: phenol–water (4:1); peak A at 6–7 cm, glycine; peak B at 13–14 cm, α-hydroxy-γ-aminobutyrate

FIG. 2. Descending paper chromatography (18 hours): EtOH–AcOH–H_2O (8:1:1); peak A at 12–13 cm, glycine; peak B at 17–18 cm, α-hydroxy-γ-aminobutyrate.

Figures 1 and 2 are radiochromatograms of aliquots of a solution obtained after the precipitation of proteins and removal of the substrate γ-hydroxyglutamate-5-C^{14} from an incubated mixture which contained a crude homogenate of rat brain tissue. The presence of radioactive α-hydroxy-γ-aminobutyrate provides evidence of decarboxylase activity in rat brain toward γ-hydroxyglutamate-5-C^{14}.

FIG. 3. The conditions of paper chromatography were those cited in Fig. 1. Single peak at 6–7 cm, glycine.

FIG. 4. The conditions of paper chromatography were those cited in Fig. 2. Single peak at 12–13 cm, glycine.

Figures 3 and 4 are radiochromatograms of aliquots of a solution obtained after the precipitation of proteins and removal of the substrate γ-hydroxyglutamate-5-C^{14} from an incubated mixture which contained a crude homogenate of rat liver tissue. There is no evidence of decarboxylase activity in the latter tissue toward γ-hydroxyglutamate-5-C^{14} as the chromatograms do not show any trace of radioactive α-hydroxy-γ-aminobutyrate. However, the presence of radioactive glycine on the chromatograms (Figs. 1 through 4) provides indirect evidence that γ-hydroxyglutamate-5-C^{14} is cleaved in rat brain, more extensively in rat liver, with the formation of radioactive glyoxylate which is then converted into glycine by amination.

Acknowledgments

The authors are indebted to the Medical Research Council (Canada) and to the Fondation Joseph Rhéaume for supporting this research.

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PRÉPARATION D'UNE PHOSPHATASE ALCALINE INTESTINALE PURIFIÉE

MICHEL LAZDUNSKI ET LUDOVIC OUELLET

Un certain nombre de méthodes ont jusqu'ici été proposées pour purifier les phosphatases alcalines de différentes origines. Les plus récentes sont celles de Lora-Tamayo (1), de Mathies (2) et de Binkley (3) pour la phosphatase alcaline du rein et celle de Portmann (4) pour la phosphatase alcaline de l'intestin. Cette note n'a pour but que de proposer une préparation relativement simple, à partir d'une substance brute disponible dans le commerce, d'une phosphatase alcaline intestinale ayant un degré de pureté comparable à celui des préparations les meilleures. À la lumière des nombreux succès obtenus par utilisation des celluloses substituées (5), la purification de la phosphatase alcaline intestinale a été tentée par chromatographie sur N,N-diéthylaminoéthyl-cellulose (DEAE).

Réactifs et Mode opératoire

La DEAE cellulose est un produit Eastman Organic Chemicals. La phosphatase alcaline utilisée provient de Mann Research Laboratories; il s'agit d'une phosphatase alcaline intestinale déjà partiellement purifiée (numéro de catalogue, 4806).

Essais d'activité

Nos essais d'activité sont sensiblement différents des déterminations classiques proposées par King (6), Bodansky (7), Roche *et al.* (8), Morton (9), etc.

L'activité est mesurée par détermination de la vitesse initiale d'hydrolyse du phosphate de *p*-nitrophénol en concentration de 10^{-3} *M*, à 15° C, dans un tampon éthanolamine (0.01 *M*)–HCl à pH 9.5 et en présence de 5×10^{-3} *M* de $MgCl_2$. La façon d'évaluer la vitesse initiale d'hydrolyse a été décrite dans une publication précédente (10). Les activités sont exprimées en moles de paranitrophénol formées par minute et par milligramme de protéine. La méthode utilisée pour la détermination du contenu en protéine des échantillons est celle de Waddell (11) dont les avantages ont été recemment mis en évidence par Murphy et Kies (12). La courbe d'étalonnage Δ (absorbance $_{215m\mu}$–absorbance $_{225m\mu}$) en fonction de la concentration de protéine a été obtenue en tampon

tris(hydroxyméthyle)aminométhane (tris) 0.1 *M*, pH 7.4. L'albumine bovine cristallisée (Mann Research Laboratories) a été utilisée comme protéine de référence. On a vérifié que l'absorbance d'une solution de phosphatase alcaline suit bien la loi de Beer dans le domaine de concentration allant de 20 à 100 μg/ml.

L'intérêt de l'utilisation du phosphate de *p*-nitrophénol est évident. La facilité d'analyse des produits est accrue puisqu'il ne s'agit que de déterminer la quantité de paranitrophénol formée, par mesure spectrophotométrique à 407 mμ. Faire le dosage à la température de 15° C se justifie par le début de désactivation observé dès pH 9.8 à température de 25° C (10).

Les chromatographies et les dialyses ont été réalisées à 4° C. La colonne utilisée a un diamètre de 29 mm. Le poids de DEAE cellulose utilisé est 25 fois celui des protéines que contient l'échantillon. La DEAE cellulose est d'abord lavée abondamment par le tampon qui servira plus tard d'éluant, puis tassée dans la colonne sous une pression relative de 0.5 atmosphère. On continue alors le lavage par le tampon jusqu'à ce que les valeurs de pH du tampon entrant et sortant soient identiques. Dans la première chromatographie, 3 grammes de phosphatase Mann dissous dans 10 ml de tris 0.01 *M* et de pH 7.4 sont adsorbés sur la colonne. L'élution est faite grâce à un gradient linéaire de concentration du tampon tris de pH 7.4 depuis 0.01 *M* jusqu'à 0.1 *M*. Les résultats obtenus sont présentés sur la Fig. 1. Les tubes 1 à 11 contiennent 25 ml de solution; 12 à 46, 15 ml; 46 à 80, 10 ml; 81 à 90, 15 ml; au-delà de

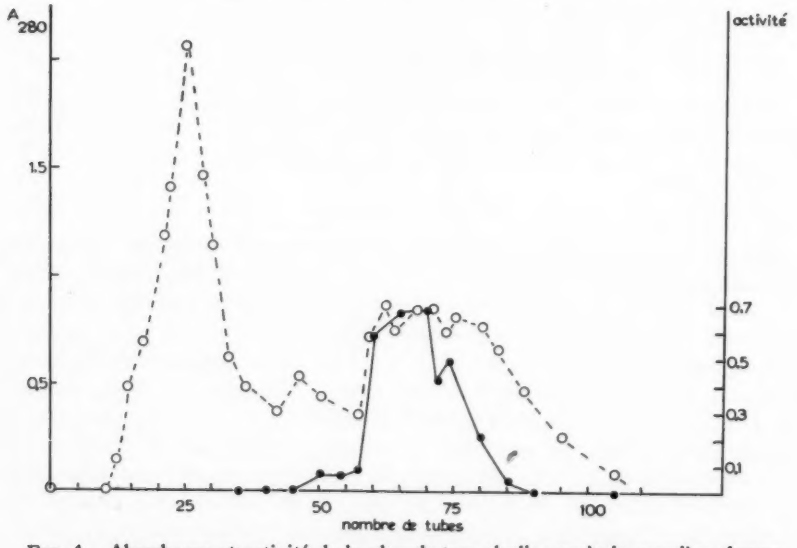

FIG. 1. Absorbance et activité de la phosphatase alcaline après la première chromatographie sur DEAE cellulose à pH 7.4. ● Activité en unités arbitraires. ○ Absorbance à 280 mμ.

90, 25 ml. L'élution par gradient n'a été utilisée que jusqu'au tube 36, on utilise ensuite le tampon tris à pH 7.4 et concentration 0.1 *M*. Chaque tube a fait l'objet d'une détermination d'absorbance à 280 mμ et d'un essai d'activité. Les activités déterminées à température ambiante sont des vitesses initiales exprimées en Δ absorbance $_{407 m\mu}$/seconde. Les fractions présentant une activité phosphatasique, contenues dans les tubes 58 à 74, sont réunies puis concentrées par lyophilisation jusqu'à un volume de 30 ml environ. Cette solution est ensuite dialysée contre un tampon tris, 0.1 *M*, +$MgCl_2$, 0.04 *M*, à pH 8.4 et 4° C pendant 24 heures. Le dialysat est alors chromatographié sur une colonne de DEAE cellulose préparée comme précédemment et l'élution est réalisée par un tampon tris, 0.01 *M*, + $MgCl_2$, 0.04 *M*, à pH 8.4 (Fig. 2).

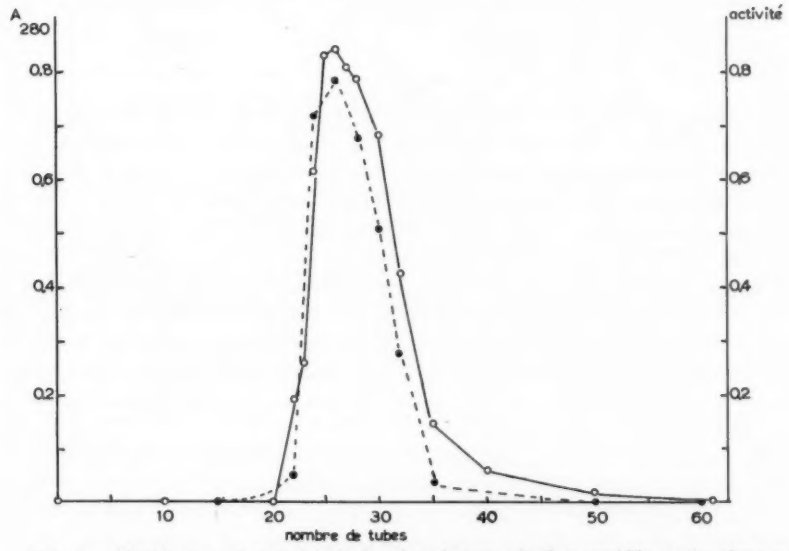

FIG. 2. Absorbance et activité de la phosphatase alcaline purifiée après chromatographie sur DEAE cellulose à pH 8.4. ● Activité en unités arbitraires. ○ Absorbance à 280 mμ.

Les quatre premiers tubes contiennent 25 ml de solution, les autres 10 ml. Il n'y a plus qu'un seul pic dans la courbe d'absorption à 280 mμ et il correspond au pic d'activité phosphatasique. Le contenu des tubes 22 à 35 est finalement dialysé contre du $ZnSO_4$ à 10^{-6} *M*, pendant 48 heures à 4° C, puis séché par lyophilisation.

Résultats et Discussion

Le Tableau I groupe les résultats obtenus en déterminant les activités après chacun des différents stades de la purification. Nos activités peuvent être transformées en unités Roche (en γ-P/min/mg N total) en les multipliant par

10^6. Nous obtenons ainsi des activités supérieures à 300,000 unités Roche dans les meilleures conditions. L'activité de notre phosphatase intestinale est donc comparable à celle de la phosphatase du rein purifiée par Binkley (3).

TABLEAU I

Activités aux différents stades de la purification

	Activité (mole de paranitrophénol/ mg protéine/minute)
Phosphatase avant chromatographie	3.44×10^{-5}
Phosphatase après première chromatographie (tubes 58 à 74)	3.43×10^{-4}
Après dialyse dans tris pH 8.4 + $MgCl_2$ 0.04 *M*	3.0×10^{-4}
Après deuxième chromatographie (tubes 22 à 35)	3.54×10^{-4}
Après dialyse contre une solution de $ZnSO_4$ 10^{-6} *M*	2.42×10^{-4}

L'activité, qui augmente d'un facteur 10 dès la première chromatographie, reste sensiblement constante au cours des deux opérations suivantes mais diminue considérablement au cours de la dernière dialyse. Lorsque la dialyse est réalisée dans l'eau distillée dans du verre, sans addition d'ions Zn^{++}, on constate, comme Van Thoai *et al.* (13), une désactivation encore plus considérable. Ceci met en évidence l'importance du zinc que Mathies a décelé dans la molécule de phosphatase rénale et dont nous pressentons la présence au sein de la molécule de phosphatase alcaline. Les récents résultats de Kunitz (14) mettent l'accent sur le rôle du pH dans la dissociation du complexe protéine–zinc. Ce complexe serait stable de pH 8 à 9, mais réversiblement dissociable à plus bas pH. L'élimination du zinc serait la cause de la désactivation observée en milieu acide.

L'électrophorèse de la phosphatase de départ, effectuée avec un appareil Perkin–Elmer à pH 9.6 dans un tampon 0.01 *M* éthanolamine + 0.1 *M* NaCl sous un courant de 10 milliampères, laisse apparaître cinq pics. Dans les mêmes conditions, l'enzyme purifiée à concentration de 7 mg/ml se déplace en un pic unique. Les résultats obtenus à pH 8.7 avec un tampon tris + 0.01 *M* NaCl sont un peu moins satisfaisants et laissent apparaître, après 5 heures, un second pic qui correspondrait à une impureté représentant peut-être 15% du poids total. Il n'est pas exclu que cette impureté puisse provenir de la partie de la phosphatase alcaline qui semble avoir été dénaturée au cours de la dernière dialyse.

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GLUCOSE AND ACETATE METABOLISM IN FISH

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In aerobic experiments, Brown (1) found that glucose-1-C^{14} and glucose-6-C^{14} were treated almost identically in whole goldfish, suggesting that glucose was being metabolized primarily through the Embden–Meyerhof scheme. He concluded that glucose metabolism in carp is not basically different from that in mammals. Blazka (2), on the other hand, set forth evidence for the thesis that anaerobic metabolism in fish ends in CO_2 and lipid. While investigating pathways of glucose oxidation in trout, I obtained some data pertaining to both of the foregoing reports.

Goldfish were anesthetized in a solution of MS_{222}, injected with about 0.5 μc of glucose (C-1 or C-6 labeled) or acetate-1-C^{14} per 15 g, and placed four at a time in a 1-liter vacuum flask containing CO_2-free water through which 100% O_2 was bubbled. The water was kept at room temperature and at about pH 6 with one or two additions of HCl (1) in order to facilitate the release of CO_2 from solution. A tube led from the sidearm of the flask to exchangeable 50-ml test tubes containing 30 ml of 1 *N* NaOH. The respiratory CO_2 was swept out into the trapping solutions, then precipitated with an excess of $Ba(OH)_2$. The $BaCO_3$ was centrifuged, washed with water, and plated directly onto weighed aluminum planchets. At the end of 12 hours, the fish were removed and killed by decerebration. Samples of liver and muscle were excised, pooled, and either placed in hot KOH for glycogen analysis (3) or minced and extracted with petroleum ether (4). Glycogen was plated directly onto weighed planchettes (5); the ether extracts were plated on lens paper (6). Radioactivity was determined with a gas flow counter (Model D-47, Nuclear Chicago).

The results of the first experiment are shown in Table I. C-1 and C-6 of glucose appeared in respiratory CO_2 at almost identical rates. However, more

TABLE I

The relative rates of metabolism of glucose-1-C^{14} and glucose-6-C^{14} by goldfish at 23° C under 100% O_2 (Four fish were injected with equal amounts of G-1-C^{14}; four, with the same amounts of G-6-C^{14})

Compound	$\frac{C^{14} \text{ from C-6}}{C^{14} \text{ from C-1}}$
CO_2	0.9
Glycogen (liver and muscle)	1.7
Fat (liver and muscle)	2.2

C-6 than C-1 was incorporated into glycogen and fat, although the deviation in the C-6/C-1 yield ratios away from unity was not great. Brown (1) reported a similar discrepancy so that on the whole these results are in excellent agreement with his.

For the anaerobic experiments, tank N_2 was bubbled through the vacuum flasks which initially contained gasless water. The water was kept at 10° C so that these experiments could be prolonged for several hours. Although temperature acclimation involves a shift in emphasis on pathways of glucose metabolism (7, 8), a short exposure to low temperature probably would not alter the picture. The tank N_2 was first passed through a train of alkaline pyrogallol to reduce the possibility of O_2 contamination. The gas leaving the chamber was examined with the Scholander analyzer (9). Maximum contamination was in the order of 0.5% to 0.7% by volume.

Figure 1 shows the rate of oxidation of glucose-1-C^{14} under anoxic and aerobic conditions. From the slope of the lines, it is apparent that a change to aerobic

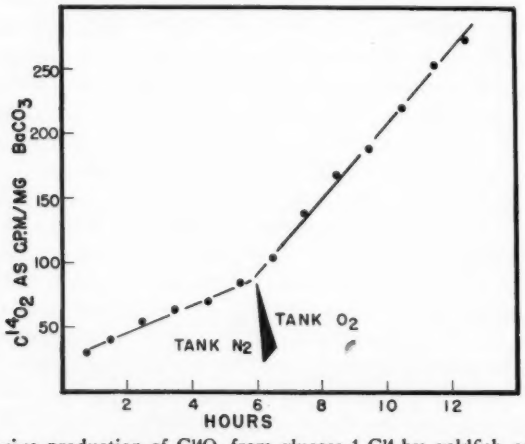

FIG. 1. In vivo production of $C^{14}O_2$ from glucose-1-C^{14} by goldfish at 10° C under anaerobic and aerobic conditions. To the left of the arrow, maximum O_2 contamination was 0.6% by volume.

conditions increased the rate of oxidation to three times the anaerobic rate. Figure 2 summarizes similar experiments using acetate-1-C^{14} as substrate instead of glucose. Here the rate of $C^{14}O_2$ production is the same under anoxia as under O_2, thus establishing conclusively that CO_2 is one end product of anaerobic metabolism in fish, as first suggested by Blazka (2). This conclusion is also supported by some earlier work (10) which showed that lactic acid is not accumulated by goldfish during anoxia. However, Prosser *et al.* (10) did not identify the metabolites being produced.

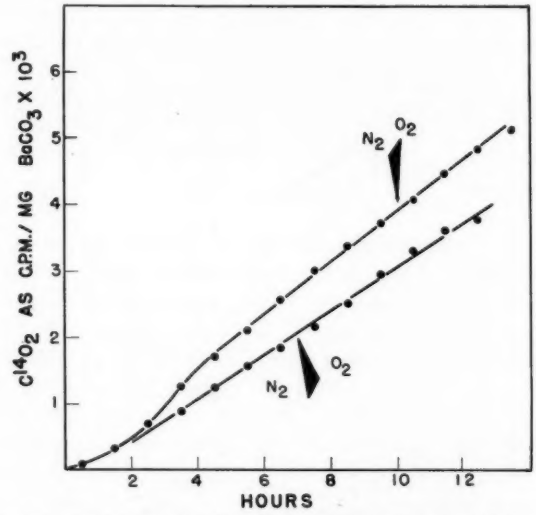

FIG. 2. In vivo production of $C^{14}O_2$ from acetate-1-C^{14} by goldfish at 10° C under anaerobic and aerobic conditions. To the left of the arrows, the maximum amount of O_2 was 0.5% and 0.7% by volume for the lower and upper curves respectively.

The invertebrate field has been extensively reviewed by von Brand (11), who believes that the main anaerobic reaction scheme is the familiar Embden–Meyerhof path. That this is likely to be the case in fish is supported by some of my experiments with trout tissues.

Warburg-type flasks were used in these experiments. About 0.25 μc of glucose (C-1 or C-6 labeled) was delivered to each flask with a syringe microburette. In each, the main chamber contained 3 ml of phosphate buffer (12) and about 0.3 g tissue, excised from trout, *Salvelinus fontinalis*, acclimated for over 2 weeks to 4° C. The tissue was sliced freehand with a razor, and all preparation was at 4° C. The center well contained a wick of filter paper in 0.2 ml of CO_2-free 20% KOH. The gas phase was either 100% O_2 or 100% N_2. In the latter case, several crystals of pyrogallol were placed in the KOH, and the preparation was incubated for 30 minutes to remove any contaminating O_2, before the radioglucose was tipped in. After an incubation period of 2 hours at 20° C,

1/2 ml of 5 *N* H_2SO_4 was added to drive off respiratory CO_2 and to kill the preparation. The KOH well was emptied and washed several times with water. The CO_2 was precipitated with $Ba(OH)_2$ and treated as before. Ether extractions were made as mentioned above. Radioactivity was corrected for the amount of tissue in each flask.

Using muscle slices, the differences between the anaerobic and aerobic experiments were not marked (Table II); with the exception of aerobic $C^{14}O_2$ production, C-1 and C-6 were metabolized similarly, with C-6/C-1 yield ratios

TABLE II

The relative rates of glucose-1-C^{14} and glucose-6-C^{14} metabolism under anaerobic and aerobic conditions by tissue slices of cold (4° C) adapted *Salvelinus fontinalis*, measured at 20° C

Preparation	Gas phase	End products	$\frac{C^{14} \text{ from C-6}}{C^{14} \text{ from C-1}}$
Liver slices	O_2	CO_2	0.26 (4)
		Fat	1.54 (4)
Liver slices	N_2	CO_2	0.90 (1)
		Fat	1.02 (1)
Muscle slices	O_2	CO_2	0.58 (4)
		Fat	0.97 (5)
Muscle slices	N_2	CO_2	0.94 (2)
		Fat	1.23 (2)

NOTE: Numbers in parentheses refer to numbers of experiments with each of glucose-1-C^{14} and glucose-6-C^{14}.

being close to unity, which would be expected in the operation of an Embden–Meyerhof scheme. However, when liver slices were incubated under 100% O_2, the C-6/C-1 yield ratio for CO_2 was very much lower than 1, a result quite consistent with an activation of the pentose cycle during cold acclimation (7). With the same tissue under 100% N_2 (Table II), the rate at which C-6 and C-1 of glucose appeared in respiratory CO_2 was almost identical. Also, in anoxia, C-6 and C-1 incorporation in liver fat was the same, while in the presence of O_2 more C-6 than C-1 appeared in fat (Table II). These results support von Brand's thesis, for the first reactions of the pentose cycle are TPN-dependent and hence would not function appreciably during anoxia. At this time, the metabolism of glucose becomes explicable on the basis of a functioning Embden–Meyerhof scheme alone.

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SOME OBSERVATIONS ON THE EFFECT OF A NATURALLY OCCURRING AGENT(S) ON THE 2,4-DINITROPHENOL-INDUCED ATPase OF LIVER MITOCHONDRIA*

W. CHEFURKA

In the course of our studies on the relationship between the oxidation–reduction state of the electron transport system and the ATPase† induced by DNP (1), the observation of Potter *et al.* (2) that the mitochondrial ATPase loses its capacity for stimulation by DNP during aging was confirmed. The present communication is a report of some experiments attempting to elucidate the mechanism of this aging process.

Mouse-liver mitochondria were prepared according to Hogeboom (3) in ice-cold 0.25 *M* sucrose in a Servall refrigerated centrifuge Model RC-2 at 0° C. The mitochondrial pellet, which was isolated at 8500×*g* for 10 minutes, resuspended, and resedimented at 9500×*g* for 10 minutes, was washed three times to remove the fluffy layer and then suspended in isotonic sucrose to the desired concentration. Usually the mitochondrial concentration was equivalent to approximately 3.0 to 3.5 mg protein per ml.

The aging process was carried out in a water bath at 23° C and aliquots of the mitochondrial suspension were withdrawn and assayed for the DNP-induced ATPase.

The data in Fig. 1 indicate that the initial low level of the ATPase activity of mouse-liver mitochondria in sucrose can be stimulated about 10-fold by DNP. However, as the mitochondria age, they lose their capacity for stimulation by DNP, though in our experience, never completely.

This loss of the DNP-induced ATPase activity does not appear to be the result of mitochondrial swelling or disruption. No detectable swelling of the mitochondria under these conditions of aging could be detected by the optical

*Contribution No. 203 from The Research Institute, Canada Department of Agriculture, London, Ontario.
†Abbreviations: ATP, adenosine triphosphate; P_i, inorganic phosphorus; DNP, 2,4-dinitriphenol; EDTA, ethylenediamine tetraacetic acid; TCA, trichloroacetic acid.

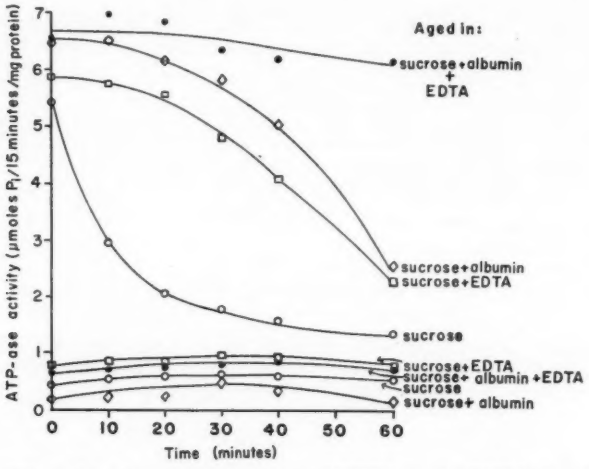

FIG. 1. Effect of bovine serum albumin and EDTA on the DNP-induced ATPase of aging mitochondria.

The mitochondria from 1 g of liver were suspended in 3 ml of cold isotonic sucrose. The aging medium was prepared by diluting the mitochondria with an equal volume of cold 0.25 *M* sucrose, EDTA (final concentration 5×10^{-3} *M*) in 0.25 *M* sucrose, bovine serum albumin (fraction V) (final concentration 0.5%) in 0.25 *M* sucrose or 5×10^{-3} *M* EDTA, serum albumin (0.5%) in 0.25 *M* sucrose. The mitochondria were then aged at 23° C for the times indicated. At the end of each aging period, 0.1 ml of the mitochondrial suspension (0.30–0.35 mg protein) was added to an assay medium which was incubated for 15 minutes. The assay medium contained EDTA 0.001 *M*, pH 7.2; KCl 0.075 *M*; Tris–acetate buffer, pH 7.2, 0.05 *M*; sucrose 0.05 *M*; and ATP 0.002 *M*, pH 7.2; the lower four curves with no DNP; upper four curves with 3×10^{-4} *M* DNP. Total volume 1.5 ml. Temperature 23° C. At the end of 15 minutes the reaction was stopped with an equal volume of 10% TCA (w/v), centrifuged, and the inorganic phosphate determined on an appropriate aliquot (12).

density method at 520 mμ. Furthermore this loss of ATPase activity also occurs in sucrose concentrations in which swelling in negligible (4). We therefore favor the view that mitochondria during aging produce an endogenous substance(s) which interferes with the stimulation of ATPase by DNP.

This hypothesis is substantiated by a further consideration of Fig. 1. Firstly, bovine serum albumin at a concentration of 0.5% not only stimulates the initial DNP-induced ATPase activity but decreases the rate at which the DNP–ATPase is lost during aging, presumably by combining with the endogenous inhibitor(s). After about 40 minutes, serum albumin at 0.5% rapidly loses its protective capacity, presumably because most of the active sites are saturated. If, however, the level is raised to 1% or 2% then serum albumin offers complete protection for the entire period of the experiment. Secondly, the loss of the DNP–ATPase is diminished by EDTA at 5×10^{-3} *M*. This is presumably due to a partial inhibition of the enzymic production of the inhibitor(s). A combination of both serum albumin (0.5%) and EDTA (5×10^{-3} *M*) offers complete protection. It is significant to note that there was only a

negligible increase in the ATPase of control mitochondria, i.e., in absence of DNP, by these agents.

The results of attempts to extract the inhibitor(s) from aged mouse-liver mitochondria are seen in Table I. It is clear that fresh mitochondria have only negligible quantities of this inhibitor(s). If, however, the mitochondria are

TABLE I

Effect of lipid factor(s) extracted from fresh and aged mitochondria on the DNP-induced ATPase of liver mitochondria

Aging medium	Assay medium	ATPase activity (μmoles P_i/15 minutes/mg protein)
Ethanol, 0.05 ml	No DPN	0.51
	DNP	4.73
Ethanol, 0.05 ml, + bovine serum albumin (1%)	No DNP	0.45
	DNP	4.94
Ethanolic extract from fresh mitochondria	No DNP	0.60
	DNP	3.63
Ethanolic extract from fresh mitochondria + serum albumin (1%)	No DNP	0.30
	DNP	4.78
Ethanolic extract from aged mitochondria	No DNP	0.08
	DNP	0.40
Ethanolic extract from aged mitochondria + serum albumin (1%)	No DNP	0.50
	DNP	3.89

NOTE: Mitochondria from five mouse livers were prepared in 0.25 *M* sucrose (approx. 15 ml) and aged for 2 hours at 35° C. They were then centrifuged at about 80,000×*g* for 10 minutes at 0° C in a Spinco model L centrifuge and extracted at 0° C in 5% acetic acid in isooctane. The isooctane was reduced to dryness and the residue washed twice with distilled H_2O. After removal of the water, the residue was suspended in 0.3 ml absolute ethanol and 0.05 ml of the ethanolic extract was added to 1 ml of cold fresh mitochondria; 0.1 ml of the mitochondrial suspension was then added to an incubation medium and the DNP-stimulated ATPase determined (see Fig. 1).

aged, a factor(s) can be extracted which, in turn, inhibits the stimulation of the ATPase by DNP of fresh mitochondria. This inhibition decreases progressively as the dilution of the inhibitor(s) increases. The inhibition is also abolished in the presence of serum albumin (1%) presumably because albumin binds the inhibitor(s). It should be noted that the inhibitor(s) has also been extracted from aged mouse-heart mitochondria and the giant mitochondria of the flight muscles of houseflies. Fresh mitochondria from these sources contained very little of the endogenous inhibitor(s). Negligible amounts of inhibitor(s) could be extracted from the supernatant of aged mitochondria suggesting that this factor(s) is produced in the mitochondria (probably at the surface) and not released into the supernatant.

The data in Table II provide further evidence for the production of an inhibitor(s) of the DNP–ATPase by aged mitochondria. Serum albumin which was aged in the presence of mouse-liver mitochondria contained an extractable substance(s) which was capable of abolishing the DNP-stimulated ATPase of fresh mitochondria. Extracts from the same quantity of fresh albumin showed negligible inhibition of the DNP-stimulated ATPase. It should also be noted that after serum albumin was aged with liver mitochondria its ability to prevent aging of fresh mitochondria was practically abolished.

TABLE II

Effect of acidic isooctane extracts of fresh and aged bovine serum albumin on the DNP-induced ATPase of mouse liver mitochondria

Aging medium	Assay medium	ATPase activity (μmoles P_i/15 minutes/mg protein)
Sucrose	No DNP	0.45
	DNP	3.54
Ethanolic extract from aged serum albumin		
Undiluted	No DNP	0.31
	DNP	0.31
Diluted 1:5	No DNP	0.46
	DNP	1.29
Ethanolic extract from fresh serum albumin		
Undiluted	No DNP	0.46
	DNP	3.34
Diluted 1:5	No DNP	0.46
	DNP	3.55

NOTE: Mitochondria from eight livers were suspended in 25 ml of 2% bovine serum albumin (fraction V) in 0.25 *M* sucrose. This suspension was then aged for 2 hours at 35° C. After centrifugation at approximately 80,000 × *g* for 10 minutes at 0° C in a Spinco model L centrifuge, the supernatant was again used as an aging medium for another batch of fresh mitochondria derived from eight mouse livers for 2 hours at 35° C. The mitochondria were again centrifuged as described above and the aged albumin was lyophilized and then extracted with acidic isooctane at 0° C. The isooctane was reduced to dryness *in vacuo* and the residue, after two water-washings and subsequent drying, was suspended in 0.25 ml absolute ethanol. The appropriate dilution of the lipid material was made with absolute ethanol; 0.05 ml of the ethanolic extract was then added to 1 ml of fresh cold mitochondria. The DNP-induced ATPase activity was determined as described in the legend of Fig. 1. An equal quantity of fresh serum albumin was extracted with acidic isooctane as controls.

It is clear from these data that a lipid substance(s) produced during aging of mitochondria is capable of preventing the "unmasking" of the mitochondrial ATPase activity by DNP. Recently a number of reports have appeared suggesting that mitochondria and submitochondrial particles produce a substance(s) capable of affecting respiratory control (5, 6, 7), oxidative phosphorylation (5, 6, 8, 9), the ATP–$^{32}P_i$ exchange (5, 6, 7), Mg^{++}-activated ATPase (5, 6, 7), and mitochondrial permeability (6, 10). In most cases these preparations have been shown to contain extractable fatty acids, some of which can duplicate in vitro, to a greater or lesser extent, the active mitochondrial preparations (5, 6, 9, 13).

This reported effect of the lipid material on oxidative phosphorylation, the ATP–$^{32}P_i$ exchange, and now the DNP-induced ATPase are further suggestive evidence that perhaps these enzymic reactions of ATP are related to the energy coupling mechanism (11). However, the exact mode of action of these fatty acids on these enzymic reactions is as yet unknown. This aspect of the problem is currently under investigation.

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